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**Spontaneous mutagenesis
in stressed
*Escherichia coli***

A thesis submitted for the degree of Doctor of Philosophy

In Genetics

By

Andrew Robert Timms BSc

The Open University

April 1998

Author no: P9276340
Date of award: 16th July 1998

This research has been conducted at the
Medical Research Council, Cell Mutation Unit
under the supervision of Professor B.A. Bridges.

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Declaration

**The work presented in this thesis is my own,
except where otherwise stated and has never been submitted to
any other university for any other degree.**

Andrew Robert Timms

April 1998

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I would like to thank everyone at the Cell Mutation Unit for their help.

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over the past four years

and Francis for taking the hard work out of media preparation.

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and Shelley Mathias for making the first

few years at the CMU fun.

Dedication

To my darling wife Karen

This Aeneas will never set sail without his Dido

“‘It is quite clear,’ replied Don Quixote, ‘that you are not experienced in this matter of adventures. They are giants, and if you are afraid, go away and say your prayers, whilst I advance and engage them in fierce and unequal battle.’

As he spoke, he dug his spurs into his steed Rocinante, paying no attention to his squire’s shouted warning that beyond all doubt they were windmills and no giants he was advancing to attack. But he went on, so positive that they were giants that he neither listened to Sancho’s cries nor noticed what they were, even when he got near them. Instead he went on shouting in a loud voice: ‘Do not fly, cowards, vile creatures, for it is one knight alone who assails you.’”

From the translation, by J.M. Cohen, of
‘The Adventures of Don Quixote’

(Miguel de Cervantes Saavedra, 1604)

There should always be a place for people to tilt at windmills

a world without such space would be poorer for it.

Long may the spirit of Don Quixote live long and prosper.

Publications arising from this thesis

Timms, A. R., Dewan, K. K. & Bridges, B. A. (1995). Growth rate effects of mutations conferring streptomycin-dependence and of ancillary mutations in the *rpsL* gene of *Escherichia coli*: implications for the clustering (hypermutation) hypothesis for spontaneous mutation. *Mutagenesis*, **10**, 463-466.

Timms, A. R. & Bridges, B. A. (1996). The *tyrT* locus of *Escherichia coli* B. *J. Bacteriol.* **178**, 2469-2470.

Bridges, B. A. & Timms, A. R. (1997). Mutation in *Escherichia coli* under starvation conditions: a new pathway leading to small deletions in strains defective in mismatch correction. *EMBO J.* **16**, 3349-3356.

Timms, A. R. & Bridges, B. A. (1998). Reversion of the tyrosine ochre strain *Escherichia coli* WU3610 under starvation conditions depends on a new gene *tas*. *Genetics*, **148**, 1627-1635.

Supporting publications

The following two publications formed the initial work upon which section 3.3 of this thesis, dealing with multiple mutations in the *rpsL* gene, was based.

Timms, A. R., Steingrimsdottir, H., Lehmann, A. R. & Bridges, B. A. (1992).

Mutant sequences in the *rpsL* gene of *Escherichia coli* B/r: mechanistic implications for spontaneous and ultraviolet light mutagenesis. *Mol. Gen. Genet.* **232**, 89-96.

Timms, A. R. & Bridges, B. A. (1993). Double independent mutational events in the *rpsL* gene of *Escherichia coli*: an example of hypermutability? *Mol. Microbiol.* **9**, 335-342.

Abbreviations and symbols

3-MeA	3-methyladenine
3-MeG	3-methylguanine
5-MT	5-methyl-DL-tryptophan
8-oxo-dA	8-oxo-7,8-dihydro-2'-deoxyadenine
8-oxo-dG	8-oxo-7,8-dihydro-2'-deoxyguanine
8-oxo-dGTP	8-oxo-7,8-dihydro-2'-deoxyguanosine 5'-triphosphate
8-oxo-dGMP	8-oxo-7,8-dihydro-2'-deoxyguanosine 5'-monophosphate
Δ	deletion
μg	microgram (10^{-6} grams)
μl	microlitre (10^{-6} litres)
μm	micrometre (10^{-6} metres)
σ^s	stationary phase sigma factor
A	adenine
AP-site	apurinic/apyrimidinic site
ATP	adenosine 5'-triphosphate
<i>B. bovis</i>	<i>Babesia bovis</i> (Eukaryota; Animalia; Protozoa; Apicomplexa; Sporozoasida; Coccidiasina; Piroplasmida; Babesiidae; Babesia)
bp	base pair(s)
BER	base excision repair
<i>B. subtilis</i>	<i>Bacillus subtilis</i> (Eubacteria; Firmicutes; Bacillaceae; Bacillus)
C	cytosine

cAMP	cyclic adenosine 5'-monophosphate
cfu	colony forming unit
CM	chorismate mutase
CPD	cyclobutane pyrimidine dimer
CRP	cAMP receptor protein
d	2'-deoxyribo
DNA	deoxyribonucleic acid
<i>E.coli</i>	<i>Escherichia coli</i> (Eubacteria; Proteobacteria; gamma subdivision; Enterobacteriaceae; Escherichia)
<i>E.herbicola</i>	<i>Erwinia herbicola</i> (Eubacteria; Proteobacteria; gamma subdivision; Enterobacteriaceae; Erwinia)
Exo I	DNA exonuclease I
Exo VII	DNA exonuclease VII
IGP	indole-glycerol phosphate
IPTG	isopropyl β -D-thiogalactopyranoside
Jm ⁻²	UV fluence (Joule(s) m ⁻²)
kb	kilobase pairs (10 ³ base pairs)
G	guanine
^o G	oxidised guanine repair system
GTP	guanosine 5'-triphosphate
l	litre
Leu	leucine
m	metre
M	molar (mole(s) l ⁻¹)

Mb	megabase pairs (10^6 base pairs)
ml	millilitre (10^{-3} litres)
mm	millimetre (10^{-3} metres)
mM	millimolar (10^{-3} mole l^{-1})
MMR	methyl-directed mismatch repair
mRNA	messenger RNA
N	either A, C, G or T bases
NER	nucleotide excision repair
ng	nanogram (10^{-9} grams)
nm	nanometre (10^{-9} metres)
NTP	nucleoside 5'-triphosphates
O⁴-T	O⁴-methylthymine
O⁶-G	O⁶-methylguanine
ORF	open reading frame
<i>P.aeruginosa</i>	<i>Pseudomonas aeruginosa</i> (Eubacteria; Proteobacteria; gamma Subdivision; Pseudomonas)
PCR	polymerase chain reaction
Phe	phenylalanine
P-protein	product of <i>E.coli pheA</i> gene (chorismate mutase and prephenate dehydratase)
Pol I	DNA polymerase I
Pol II	DNA polymerase II
Pol III	DNA polymerase III holoenzyme
RNA	ribonucleic acid

ROS	reactive oxygen species
rRNA	ribosomal RNA
Sm ^D	streptomycin-dependent
Sm ^R	streptomycin-resistant
Sm ^S	streptomycin-sensitive
SOS	inducible DNA repair regulon
SSCP	single stranded conformational polymorphism analysis
T	thymine
Tn	transposon
T-protein	product of <i>E.coli tyrA</i> gene (chorismate mutase and prephenate dehydrogenase)
tRNA	transfer RNA
Trp	tryptophan
Tyr	tyrosine
u	unit(s)
U	uracil
UV-A	ultra violet light (400-320nm)
UV-B	ultra violet light (320-290nm)
UV-C	ultra violet light (290-100nm)
VSP	very short patch mismatch repair

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Abstract

Spontaneous mutagenesis in stressed *Escherichia coli*

Mutation processes occurring in starving or stressed bacteria are different from those that are found in growing bacteria. The mutagenic response of *E. coli* to a number of different stress conditions, including amino acid starvation and challenge with the antibiotic streptomycin has been examined. In an *E. coli trpA23* strain starved for tryptophan, the formation of small in-frame deletions was stimulated by the persistence of oxidatively damaged guanine and unrepaired mismatches in the DNA. Most (75%) of the deletions had direct repeats at each termini, one of which was lost in the deletion process. The deletions restored functionality to the product of the *trpA* gene enabling the mutants to form slow growing colonies on minimal plates lacking tryptophan.

During starvation of an *E. coli tyrA14* strain WU3610 for the amino acid tyrosine, a new phenotypic suppressor gene (*tas*) was isolated that specifically complemented the absence of prephenate dehydrogenase activity. The gene is necessary for the process of starvation-associated mutagenesis in this strain; mutants do not arise during starvation in a *tas* deletion strain.

A high proportion (up to 20%) of ancillary mutations within the *rpsL* gene of *E. coli* have previously been found in newly arising streptomycin dependent mutants and have been shown to confer a selective advantage in established strains. This study shows that they also confer an advantage in mutants containing mixed wild type and streptomycin dependent ribosomes in the presence and absence of streptomycin. However, this selective advantage is still not enough to account for the rate at which these ancillary mutations are recovered and we need to assume a mutation rate of 10^{-3} to 10^{-4} /genc/replication to explain the observed frequency.

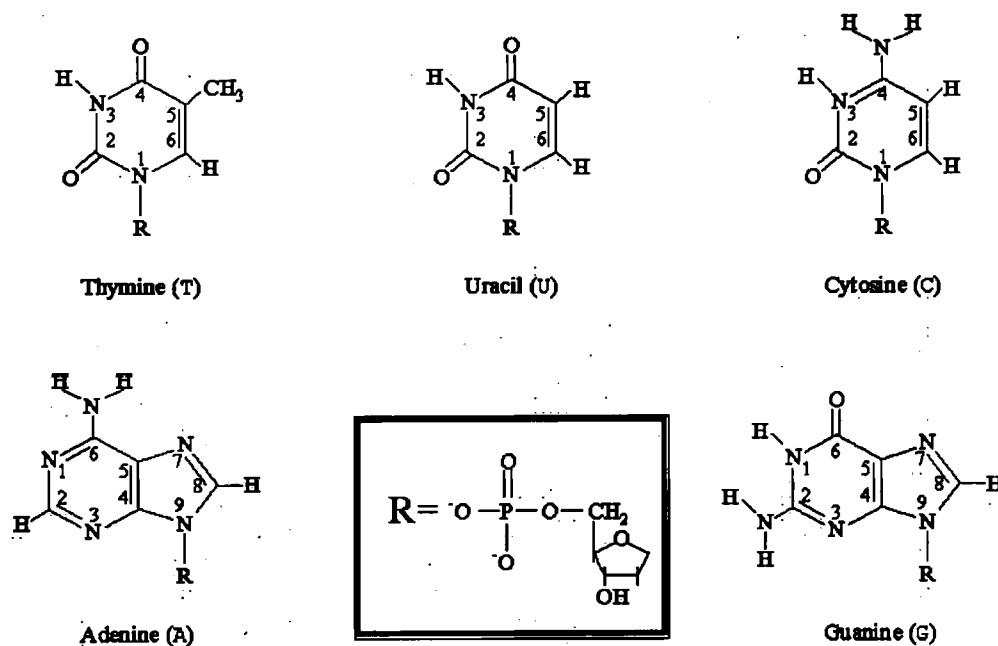
1 Introduction

Heritable genetic information is stored as a sequence of five nucleotide bases adenine (A), cytosine (C), guanine (G), thymine (T) and uracil (U) whose structures are shown in Figure 1.1. In the majority of organisms and exclusively in higher organisms the information storage molecule is deoxyribonucleic acid (DNA) and does not normally include uracil. The information, or genetic code, stored as a sequence of the four bases along a DNA strand is ultimately interpreted by the transcriptional and translational machinery of the cell to form the structural and catalytic molecules necessary for life.

Storage molecules and the information contained within them need to be maintained and accurately replicated for life to continue and for organisms to reproduce. However, even in organisms where fidelity and repair processes are fully functional there is still a small probability that errors will be introduced into the genetic material, a process termed spontaneous mutagenesis. Under most conditions, bacteria can reduce this spontaneous error rate to around 10^{-10} mutations/base pair/replication (Drake, 1991a; 1991b) although many factors can influence this figure. While a full review of spontaneous mutagenesis falls beyond the scope of this thesis, the introduction will highlight some of the causes of, and mechanisms that bacteria use to reduce the effects of spontaneous mutagenesis. For more detailed accounts see reviews by Friedberg *et al.* (1995), Hutchinson (1996) and Miller (1996).

Figure 1.1

Structure of the five nucleobases



Structures of the five bases commonly found in nucleic acids, thymine (T), uracil (U), cytosine (C), adenine (A) and guanine (G). When the group R = deoxyribose 5'-monophosphate the compounds become the corresponding nucleotides; thymidine (TMP), uridine (UMP), cytidine (CMP), adenosine (AMP) and guanosine (GMP) deoxyribose monophosphates. Under normal conditions, uracil is not incorporated into DNA but may be formed *in situ* by chemical processes. It is however, incorporated into RNA in place of thymine.

Errors in the genetic material can arise from three main sources i) damage, both physical and chemical, to the nucleotide bases or nucleic acid structure, ii) replicative errors introduced by polymerases copying the genetic material or iii) errors in

recombination and repair processes. While many DNA repair processes such as nucleotide excision repair and mismatch repair have counterparts in higher organisms the focus of this introduction will be exclusively on bacteria.

Most of the information about mechanisms and effects of mutagenesis in bacteria has been obtained from cells that are actively growing *i.e.* during the logarithmic growth phase. In the last ten years, focus has increasingly switched to the processes occurring in bacteria that are starving or under some other form of physiological stress, perhaps a situation more akin to that experienced by bacteria in natural environments. The process of stationary phase mutagenesis will also be discussed in the latter part of this introduction. The mutagenic effects of stress situations on bacteria are also considered in the results sections of this thesis where three quite different responses to adverse conditions are described. Finally, while a high rate of spontaneous mutations would ultimately be deleterious to an organism the evolutionary process requires there to be genetic variation between individuals. The evolution and role of mutator alleles in populations of *E.coli* have been assessed both experimentally and theoretically (Sniegowski *et al.*, 1997; Taddei *et al.*, 1997c). Populations containing mutators evolve much faster than "normal" populations and there may be selective pressure for the evolution of bacteria with increased mutator activity during periods of stress or extreme selection. Once mutator activity is present, there is some selective pressure for its maintenance, providing that the deleterious effects of increased mutation rate does not exceed the benefit gained by increased rate of adaptation (Mao *et al.*, 1997; Sniegowski *et al.*, 1997).

1.1 Origins of spontaneous mutations

1.1.1 Chemical and physical modification to DNA structure

As well as external mutagenic chemicals entering the cell and causing damage to nucleotides, normal metabolism may also be a major source of intracellular mutagens. Sargentini and Smith (1986) found that the metabolism of the amino acid phenylalanine could be mutagenic and that the process was dependent on error-prone repair. Metabolism of some other amino acids has also been shown to give rise to endogenous mutagens (Quinones and Piechocki, 1985; Glatt, 1990). As well as amino acids, sugar metabolism may give rise to compounds that interact and modify DNA bases (Lee and Cerami, 1990) again potentially leading to mutagenesis.

Oxidative metabolism gives rise directly or indirectly to a number of highly reactive and potentially mutagenic species such as hydrogen peroxide (H_2O_2), superoxide radical (O_2^-), singlet oxygen ($^1\text{O}_2$) and the hydroxyl radical (OH^\cdot), collectively known as reactive oxygen species (ROS). These can react with DNA, in reactions often mediated by metal ions such as iron, to modify bases and produce abasic sites or strand breaks (Yu and Anderson, 1997). The oxidised base analogue that has received most attention is 8-oxo-7,8-dihydro-2'-deoxyguanine (8-oxo-dG) which has a similar propensity to pair with adenine as with cytosine (Wood *et al.*, 1990; Shibutani *et al.*, 1991; Moriya *et al.*, 1991) and can ultimately result in G:C→T:A transversions. Considering the effort that bacteria invest in repair systems to deal

with the effects of oxidatively damaged guanine (three separate enzymes), perhaps the cell also views 8-oxo-dG as particularly worthy of attention.

ROS also attack nucleotide bases other than guanine, the major oxidised product of A is 8-oxo-7,8-dihydro-2'-deoxyguanine (8-oxo-dA), however, this product largely pairs with T and so is not considered to be mutagenic (Shibutani *et al.*, 1993). The most common oxidised form of thymine is thymine glycol which can block some polymerases and can thus be toxic in the absence of excision repair but is relatively non-mutagenic as it mainly pairs with A (Basu *et al.*, 1989; Hayes *et al.*, 1988). Oxidisation of cytosine can lead to the formation of 5-hydroxycytosine, and of uracil to 5-hydroxyuracil and uracil glycol (Wagner *et al.*, 1992). *In vitro* these 5-hydroxypyrimidines can mispair with adenine or cytosine giving rise to C:G→T:A transitions or C:G→G:C transversions. A wide range of ROS induced mutations have been found in various systems including base substitutions, frameshifts, deletions and insertions (McBride *et al.*, 1991; Tchou and Grollman, 1993; van den Akker *et al.*, 1994; Ono *et al.*, 1995).

As with all chemical systems the component bases of DNA are in a state of constant flux. This can lead to spontaneous alterations of their chemical structures and in some cases to mutagenic hotspots. Each of the four bases of DNA can exist as two tautomeric forms (Watson, 1976). Guanine and thymine normally exist in the keto form (C=O), at the C-6 and C-4 positions respectively, but they can infrequently change to enol tautomers (C-OH). Similarly, adenine and cytosine form either amino (NH₂) or imino (NH) forms at the C-6 and C-4 positions respectively, with the amino

form being dominant. Under normal physiological conditions, the enol and imino forms are rare but transitions between the two tautomers of each base do occur spontaneously. The enol form of guanine pairs well with thymine and *vice versa*, while the imino form of cytosine pairs with normal adenine and *vice versa*. If a base happens to be in the rare form when it encounters a replication fork, the probability of mispairing is high and a mutation is likely to result.

A number of alterations to the DNA primary structure including formation of apurinic/aprimidinic (AP) sites and cyclobutane pyrimidine dimers or pyrimidine-pyrimidone (6-4) photoproducts can lead to the formation of pre-mutagenic sites. AP-sites result from the cleavage of the bond linking the base to the deoxyribose in the sugar phosphate backbone of DNA. They can occur spontaneously or after alkylation of the base, which weakens the bond linking base and backbone and increases the probability of spontaneous base loss (Loeb and Preston, 1986). They are also formed as intermediates by some repair enzymes *e.g.* MutY, 3-methyladenine-DNA-glycosylase and uracil-N-Glycosylase. Lindahl (1979) has calculated that the spontaneous rate of depurination is approximately one per *E.coli* genome/hour, assuming one replication per hour. Depyrimidation occurs less frequently at about $1/20^{\text{th}}$ that of the rate of depurination. AP-sites are non-coding lesions and are pre-mutagenic sites usually resulting in base substitutions but also leading to base deletion or addition.

Cyclobutane pyrimidine dimers (CPD) are formed by covalent linkage between two adjacent pyrimidines TT, CT, TC or CC and are induced upon exposure of the DNA

to ultra violet (UV) light. Studies have indicated that these lesions are induced, by 254nm UV, in approximate ratios of 68:13:16:3 with TT dimers being the majority and CC dimers occurring relatively infrequently (Friedberg *et al.*, 1995). The UV portion of the spectrum has been split into three wavelength bands, UV-A (400 to 320nm), UV-B (320 to 290nm) and UV-C (290 to 100nm). While most studies have utilised 254nm UV light to introduce lesions in DNA, this portion of the spectrum is not relevant to spontaneous mutagenesis, as UV-C is effectively filtered out by the atmosphere. However, some UV-B and most UV-A wavelengths do reach the earth's surface and will induce similar photolesions in DNA, only less efficiently than do UV-C wavelengths.

In some situations, CPD's can be highly lethal. In the absence of an induced SOS response and in an excision repair defective strain, a single CPD in single stranded M13 based vectors effectively blocked survival of 99.5% of the DNA molecules (Banerjee *et al.*, 1988; 1990). Upon SOS induction, a 50-100 fold increase in survival was seen. Lethality is thought to be due to direct blockage of the replication fork by the photodimer (Banerjee *et al.*, 1988). However, Rupp (1996) has advanced the hypothesis that DNA replication forks can proceed past many photodimers before coming to a halt. A consequence of bypass in double stranded DNA is the production of single strand gaps, which are then subject to post replicative recombination repair and synthesis. Whether the polymerase physically dissociates from the DNA strand and reinitiates downstream from the dimer or whether it continues past the dimer without active polymerisation is unknown. In either case, the observed lethality is thus the result of processing or repair of the structures created by bypass events.

Despite the efficiency with which CPD's can interfere with replication they do not appear to be highly mutagenic, with only seven percent of bypass events in SOS induced cells leading to a mutation (Banerjee *et al.*, 1988). However, the mutations obtained were targeted to the site of the lesion and consisted of single base deletions, T→A transversions and T→C transitions.

Another UV induced lesion is the pyrimidine-pyrimidone (6-4) photoproduct. These lesions have mainly been found at TC sequences and less frequently at CC and at TT sequences but cannot be detected at CT sites. In general, they are induced less frequently than cyclobutane pyrimidine dimers in most sequence contexts but in contrast to CPD's, (6-4) photoproducts are highly mutagenic with up to 90% of bypass events resulting in a mutation (LeClerc *et al.*, 1991; Horsfall and Lawrence, 1994). (6-4) Photoproducts can exist as two different isomers the so-called normal isomer and the Dewar valence isomer, both of which can be produced by UV irradiation (Douki *et al.*, 1991; Douki and Cadet, 1992). The mutational spectra produced by these two forms of (6-4) photoproduct are different and illustrate that the chemical nature of the lesion influences the nature of the mutations that are produced. The normal isomer is extremely mutagenic with 91% of bypass events resulting in a targeted mutation of which 93% were 3' T→C transitions. The Dewar isomer was less mutagenic, only 53% of bypass events resulting in a mutation, and less specific in the mutations produced 46% being 3' T→C transitions (LeClerc *et al.*, 1991).

Three of the four bases in DNA contain amino groups. These bases; adenine, guanine

and cytosine and its modified derivative 5-methylcytosine can spontaneously lose their amino groups to form hypoxanthine, xanthine, uracil and thymine respectively (Lindahl, 1979; 1993). The resulting base analogues can give rise to errors from mispairing with deamination of cytosine to uracil, producing a G:U mismatch, being the most frequent event. Subsequent replication may then produce a G:C→A:T base pair transition by virtue of the pairing affinity of uracil for adenine. Cytosine deamination is known to be of biological importance since *E.coli* deficient in removal of uracil from DNA exhibits increased spontaneous mutation frequencies (Duncan and Weiss, 1978). In single-stranded DNA the half-life of a cytosine residue was calculated as about 200 years (*i.e.* a rate of around $2 \times 10^{-10}/s$). The rate of cytosine deamination in duplex DNA is only about 1% of that in single stranded DNA (Lindahl and Nyberg, 1974) so in the *E.coli* chromosome consisting of 4.7Mb and assuming 50% G:C content a spontaneous deamination may occur every 200,000 seconds $(2 \times 10^{-10} / 2.35 \times 10^6) / 100$ or one event every 55 hours. Transcription, replication and spontaneous DNA denaturation or “breathing”, where DNA transiently exists in the single stranded state, would all be expected to affect the localised rate of cytosine deamination. Some evidence for this comes from the work of Fix and Glickman (1987) where 77% of the G:C→A:T transitions (presumably produced by deamination of cytosine) occurred in the non-transcribed strand of the *lacI* gene in an *ung* strain of *E.coli*. Some of the cytosine residues (about 0.75%) in *E.coli* exist in the modified form 5-methylcytosine resulting from the action of the Dcm methylase. Upon deamination this base analogue forms thymine giving rise to a T:G mispair. These mispairs appear to be less efficiently repaired than other mispairs and can eventually result in C:G→T:A transitions (Duncan and Miller, 1980).

5-methylcytosine is the site of mutational hotspots in the *lacI* gene of *E.coli* and *cI* in λ prophage (Coulondre *et al.*, 1978; Lieb, 1991) and so is biologically important as a source of mutations.

The deamination of adenine and guanine bases also occurs but at a rate very much lower than that for cytosine. Deamination of adenine would lead to the formation of hypoxanthine giving the potential for A:T→G:C transitions (Lindahl, 1979). Xanthine arising from deamination of guanine would not appear to code for either cytosine or thymine and so must be considered a non-instructive DNA lesion leading to error-prone bypass of the lesion and a range of mutational events.

Methylation of bases other than cytosine can lead to mutation. *S*-Adenosylmethionine is a donor of methyl groups for a number of biosynthetic pathways in *E.coli* including the methylation of adenine and cytosine in DNA by Dam methylase and Dcm methylase respectively. It also has the potential to alkylate other bases forming 3-methyladenosine and 7-methylguanine (Rydberg and Lindahl, 1982) although these do not appear to be mutagenic. Other highly mutagenic alkylated products include O⁶-methylguanine leading to O⁶-G:T mispairs and G:C→A:T transitions and O⁴-methylthymine giving O⁴-T:G mispairs leading to T:A→C:G transitions also occur but the mechanism of their formation does not appear to involve *S*-adenosylmethionine (Lindahl, 1993).

Finally, while not strictly considered as a form of damage it is necessary to consider the effect of primary and secondary structure on DNA replication and mutagenesis.

The information contained in DNA is stored as a sequence of the four bases A, C, G and T, which form the primary structure of the two strands composing the double helix. However, the DNA molecule can adopt higher order or secondary structure depending on for example local sequence or supercoiling of the two strands. The level of supercoiling is affected by a number of proteins including the histone like proteins, H-NS and HU, and the DNA topoisomerases such as DNA gyrase. Strains deficient in *hnsA*, which codes for H-NS, show an increased mutator activity specifically for long deletions (Lejeune and Danchin, 1990) the formation of which is *recA* independent.

The DNA duplex normally exists in the right-handed B-form double helix under physiological conditions. The two chains run antiparallel to each other with the sugar-phosphate backbones orientated in the 5' to 3' direction in each strand. Each turn of the double helix contains 10.5 base pairs per turn and the nucleotides in B-form DNA are exclusively in the *anti*-conformation with respect to the orientation of the *N*-glycosidic bond between base and sugar. The DNA double helix can adopt two other recognised conformations; the A-form which is probably not relevant under physiological conditions and the Z-form which does occur in some contexts.

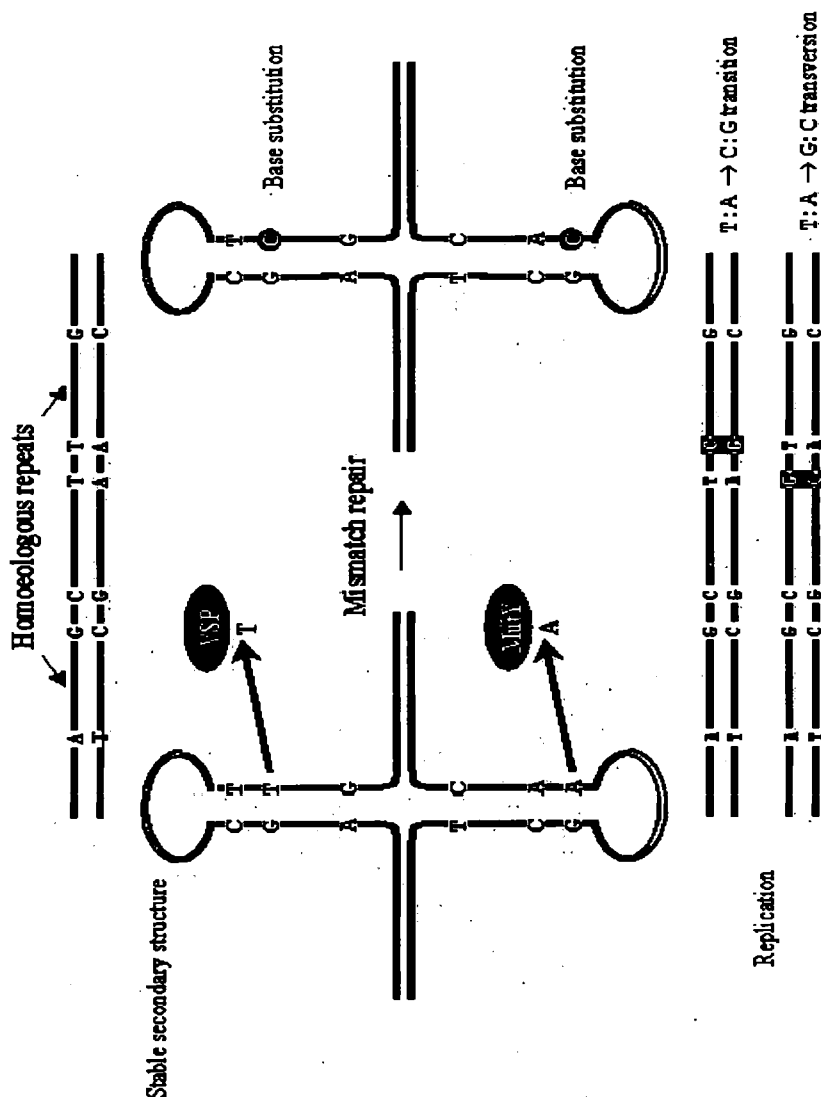
The left-handed Z-form of DNA differs from the B-form in having a 12 base pair helix repeat and the bases have alternating *anti* and *syn* conformations about the base-sugar bond. The Z-form is favoured by sequences containing alternating purine and pyrimidines bases, preferentially at CG repeat sequences although AT sequences have also been shown to adopt this structure (McLean *et al.*, 1986). Change in

conformation is promoted by increased superhelicity, with the Z-form effectively reducing the local superhelicity of the DNA. The formation of Z-form DNA may have a number of consequences among which is the increased accessibility of certain atoms in the bases, for example O⁶, N⁷ and C-8 positions in guanine. This may increase the availability of these atoms as alkyl group acceptors or to attack by oxidative species. A high incidence of deletions has also been observed near to blocks of alternating CG sequences (Freund *et al.*, 1989; Bichara *et al.*, 1995) which may be related to local secondary structure formation.

Where palindromic sequences occur they can lead to the formation of hairpins and when these occur simultaneously in both DNA strands this is termed a cruciform structure. Processes of hairpin formation initiation are uncertain although it is thought to be the result of a centre dependent mechanism (Chalker *et al.*, 1993; Davison and Leach, 1994a; 1994b). Processing of hairpins for example by SbcDC or RuvC endonucleases could lead to structures prone to deletion formation, for example by formation of pseudo-single-stranded regions of DNA after degradation of a hairpin structure on one strand of the duplex leaving the other hairpin intact. Processing of hairpins by SbcDC requires RecA or RecBCD functionality for viability (Leach *et al.*, 1997) suggesting a recombinational mechanism. Alternatively, repair of mismatches in pseudopalindromes could lead to point mutations if they were subject to repair by one of the mismatch repair systems **Figure 1.11.1.**

Figure 1.11.1

Repair of pseudopalindromes to produce base substitution mutations



Pseudopalindromes may form secondary structures *in vivo*, depending on parameters such as thermal stability or frequency of DNA replication or transcription. Pairing of heterologous sequences will create mismatches, which can be substrates for a variety of repair mechanisms *e.g.* MMR, MutY or VSP. Repair of such mismatches, that do not destabilise the secondary structure, could result in point mutations occurring during subsequent rounds of replication or recombination.

As well as palindromes (also known as inverted repeats) other sequences such as tandem repeats, interspersed direct repeats, simple repeat sequences or trinucleotide repeat sequences have also been linked to mutagenesis (Albertini *et al.*, 1982a; 1982b; Levinson and Gutman, 1987a; Ji *et al.*, 1996; Wells, 1996). Representations of potential deletogenic mechanisms are shown in Figure 1.11.2. Direct repeats can lead to template switching or copy choice errors by the replicating polymerase (Kunkel, 1990; Bi and Liu, 1996) a process that mostly results in deletion formation. Unlike normal recombination, this process is independent of almost all recombination functions, requires minimal homology and displays a distance dependent frequency of recombination (Bi and Liu, 1994). Direct repeats may also provide alternative regions of homology during repair of double-strand breaks by RecBCD when single-stranded regions of DNA are produced by degradation from double-strand blunt ends.

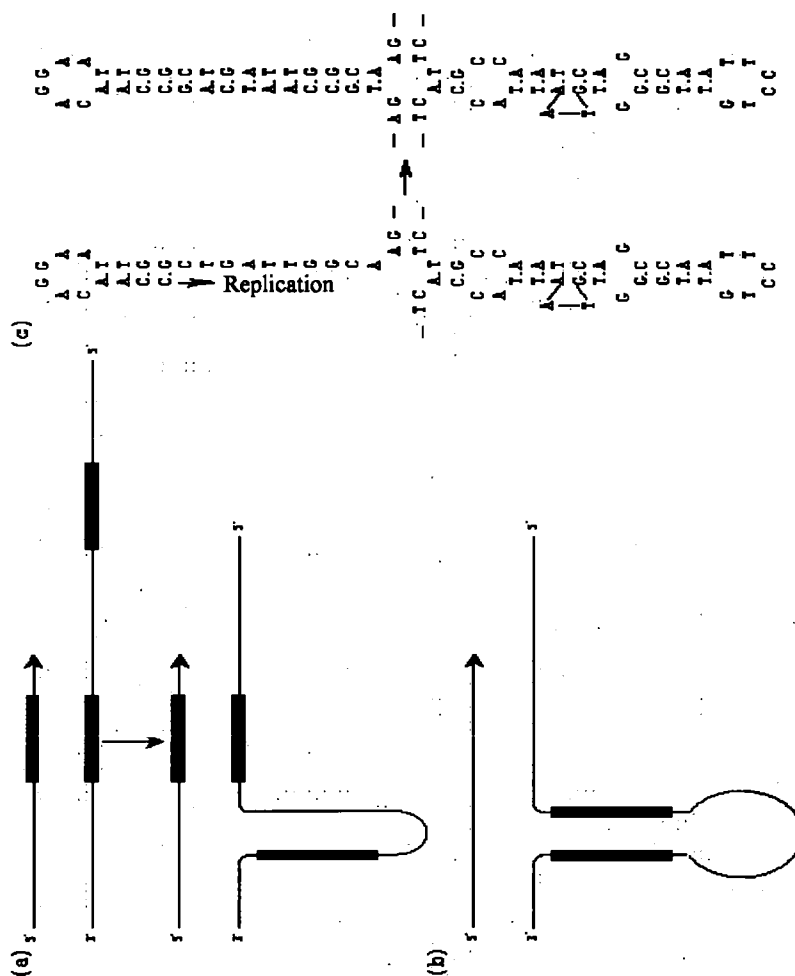
1.12 Polymerase errors

Most replicative synthesis of the bacterial chromosome is performed by DNA polymerase III holoenzyme (Pol III) with the polymerase α subunit coded for by the *dnaE* gene. However, approximately one in every 10^3 to 10^4 incorporations performed by Pol III results in a mismatched base pair (Petruska and Goodman, 1985; Perrino and Loeb, 1989). This is far higher than the 10^{-9} to 10^{-10} errors/base pair replicated measured *in vivo* and suggests that there are mechanisms other than selection of the correct nucleotide to maintain replication fidelity. Pol III is a highly processive enzyme and achieves a synthesis rate of about 1000 nucleotides/second.

Putative deletion mechanisms involving repeat sequences

Putative deletogenic mechanisms involving a) direct repeat sequences subject to misalignment during replication and involving the formation of a deletion encompassing one of the repeat sequences. b) Inverted repeats forming a stable hairpin structure on one or both strands. Processing of the hairpins may lead to deletion of one or both repeats. c) Pseudopalindromic DNA forming a substrate for initiation of DNA synthesis. The result of which is two perfect-inverted repeats that may be subject to further repair or processing. The perfect palindromes may have increased thermal stability enhancing their ability to participate or initiate deletogenic events. Re-pairing of the newly synthesised region with the sequence on the complementary strand of the duplex DNA will produce mismatches which may themselves be subject to mismatch correction, perhaps producing base substitutions or deletions. Adapted from Hutchinson, 1996.

Putative deletion mechanisms involving repeat sequences



16

transitions (Halliday and Glickman, 1991) and this bias appears to occur at the base insertion level (Schaaper, 1993). Correct base pairing could be selected for at the transition state if an incorrectly paired dNTP were to dissociate rapidly before polymerisation or if there were slower phosphodiester bond formation for misoriented bases.

Despite this stringent base-pairing requirement, base pair selection alone is not sufficient to account for the high fidelity of Pol III. A significant contribution to fidelity comes from other components of the holoenzyme. A second line of defence consists of the 3'→5' exonucleolytic or "editing" function of DNA polymerase III holoenzyme, coded for by the *dnaQ* gene. This ϵ -subunit removes bases from the growing strand that do not pair with the template and is probably helped by the fact that chain elongation is slowed by a misinserted nucleotide (Petruska *et al.*, 1988; Perrino and Loeb, 1989). A possible discrimination mechanism for the editing exonuclease is the melting capacity of the mispaired DNA, which will be lower than that of fully complementary sequences (Brutlag and Kornberg, 1972; Petruska and Goodman, 1985). Proof-reading activity reduces the error rate to about 10^{-4} to 10^{-6} errors/base pair replicated and interestingly has a stronger discrimination against transversions, as does the base insertion step of DNA polymerisation by Pol III (Schaaper, 1993). Mutators *i.e.* mutants defective in DnaQ proof-reading activity can have substantial effects on the mutation rate of the cell, increasing mutation frequencies up to 10,000 fold. Most of this comes from saturation of post replicative mismatch repair (Schaaper, 1988; Schaaper *et al.*, 1989; Damagnez *et al.*, 1989; Schaaper and Radman, 1989; Fijalkowska and Schaaper, 1996), which removes any

mispaired bases after replication, rather than as a direct consequence of inactive polymerase proof-reading (Fijalkowska and Schaaper, 1995).

Obviously, factors affecting either the polymerase α subunit DnaE or the proof-reading function DnaQ will affect the mutation rate of the host. Transient mutators could be produced by transcriptional or translational errors, which can occur at a frequency as high as 10^{-4} to 10^{-5} /incorporation during translation (Parker, 1989) and could occasionally produce an altered polymerase. Because the mutator polymerases are produced occasionally at the mRNA or protein synthesis levels they are not heritable hence the term transient (Ninio, 1991a; 1991b). Erroneous DnaQ molecules could be produced at as high a level as 20 per 10^4 molecules (Miller, 1996). Ninio (1991b) has estimated that such transient mutators could account for up to 10% of all spontaneous base substitutions.

E.coli contains two other polymerase apart from Pol III, these are called Pol I and Pol II. Pol II will be dealt with further in the section on stationary phase mutation. Pol I is the polymerase responsible for much of the DNA repair synthesis in *E.coli* for example resynthesis of excision tracts produced by MMR or NER mechanisms. A correlation has been made between the processivity of DNA polymerases and their accuracy (Kunkel and Bebenek, 1988). Pol I unlike Pol III is not processive *i.e.* will not synthesis DNA in huge tracks but tends to stall and can dissociate from the template. There is evidence that some specific sequences can act as pause sites and that these sites are regions of increased mutagenesis. In two studies using *PolAI* mutants of *E.coli* it was found that the sequences GTGG were hot spots for mutational

events including base substitutions, frameshifts, duplications and deletions (Fix *et al.*, 1987; Jankovic *et al.*, 1990). This suggests that Pol I interacts with these sites in some way and prevents mutations occurring under normal circumstances. The GTG motif may also be a pause site for chain elongation by Pol III, in *E.coli*, stimulating misalignment (Mo *et al.*, 1991). Matsui *et al.* (1996) have also suggested that some activated metabolic intermediates can act as site specific endogenous mutagens, targeting mutations to specific pause sites and leading to base substitutions, frameshifts and deletions.

1.13 Errors of recombination mechanisms

Sargentini and Smith (1985) concluded “recombination errors do not play a major role in spontaneous mutagenesis”, which they presumably restricted to include only base substitution mutations. However, spontaneous frameshifts and deletions do seem to result from mechanisms involving recombination and replication. Recombination plays a major role in certain DNA repair processes but can itself lead to mutations *e.g.* recombinational repair of double strand breaks is responsible for the formation of long deletion mutations (Sargentini and Smith, 1991). Inactive RecBCD results in an approximately 3-fold reduction in the rate of deletion formation (Sargentini and Smith, 1991) however neither *recA* or *recB* mutations eliminate all spontaneous deletions, especially those involving little or no homology at either terminus. The *recA*, *recB* dependent spontaneous long-deletion mutagenesis could be due to repair of double strand breaks produced metabolically, while the *recA*, *recB*-

independent production of deletions may be due to slippage and mispairing during DNA replication or repair (Ripley, 1990).

There is a *recA* dependent mode of DNA repair, initiated by nucleotide excision that is mutagenic (Nishioka and Doudney, 1970; Bridges and Mottershead, 1971) and is a component of the SOS DNA damage inducible repair system. Since *uvr⁻* strains show increased mutagenesis compared to wild type cells this suggests that post replicational repair is more mutagenic than is excision repair. Postreplication repair is a multipathway *recA* dependent process for the repair of daughter strand gaps and of the DNA double strand breaks that can arise at these gaps (reviewed in West, 1992; Eggleston and West, 1996; Lloyd and Low, 1996; West, 1997).

1.2 DNA repair

In view of the multitude of ways that the genetic information can become corrupted, it is no surprise that the cell invests a great deal of energy in repair systems to maintain the integrity of its DNA. Some repair systems such as the SOS response and nucleotide excision repair (NER) fall outside the scope of this introduction and will only be considered briefly, for reviews see; Walker, 1995 (SOS); Lin and Sancar, 1992; Sancar, 1996 (NER).

1.21 Methyl directed mismatch repair

Error reduction during replication of the chromosome has three main components, base selection and proof-reading have been described above and reduce the rate to about 10^{-6} errors/base pair replicated. However, there is a third level of postreplicative error correction and this consists of base mismatch correction, the most important component of which is long patch methyl directed mismatch repair (MMR). MMR involves the protein products of four genes *mutH*, *mutL*, *mutS* and *uvrD* which combine to repair base-base mispairs and small insertions or deletions (up to about four base pairs) generated during DNA replication (Dohet *et al.*, 1986; Learn and Grafstrom, 1989; Parker and Marinus, 1992; Carraway and Marinus, 1993; reviewed by Modrich and Lahue, 1996).

The DNA strand to be repaired is identified by virtue of the state of adenine methylation at GATC sequences (Wagner *et al.*, 1984; Kramer *et al.*, 1984; Dohet *et al.*, 1986) or by persistent strand breaks (Langle-Rouault *et al.*, 1987; Lahue *et al.*, 1989). Methylation at GATC sites occurs within a few minutes of DNA replication and is performed by the Dam methylase. However, the newly synthesised DNA is transiently hemimethylated and serves to target mismatch repair to the unmethylated *i.e.* new strand. In *dam* mutants, where neither strand is methylated, repair is not targeted to the new strand and *dam* mutants show mutator phenotypes.

The repair process begins with the recognition of the base-base mismatch by MutS. While all eight possible base-base mispairs were preferentially bound by MutS

compared to homoduplex DNA (Su and Modrich, 1986; Su *et al.*, 1988) this does not necessarily reflect the efficiency with which they were repaired. The range of efficiencies of repair of some mismatches varies considerably, depending on the specific mismatch and on the local sequence context (Jones *et al.*, 1987; Blake *et al.*, 1992). Despite this some mismatches are consistently found to be repaired more effectively than others, in particular G:T and A:C mismatches which can lead to transition mutations are very efficiently repaired (Dohet *et al.*, 1985; Leong *et al.*, 1986). This neatly complements the bias of Pol III for reducing transversion mutations (Schaaper, 1993) by its inherent base discrimination and proof-reading functions. Of the base pairs that can give rise to transversion mutations the order of repair efficiency is generally G:G, A:A > T:T, C:T and G:A >> C:C mispairs, which appear to be subject to little repair by MMR.

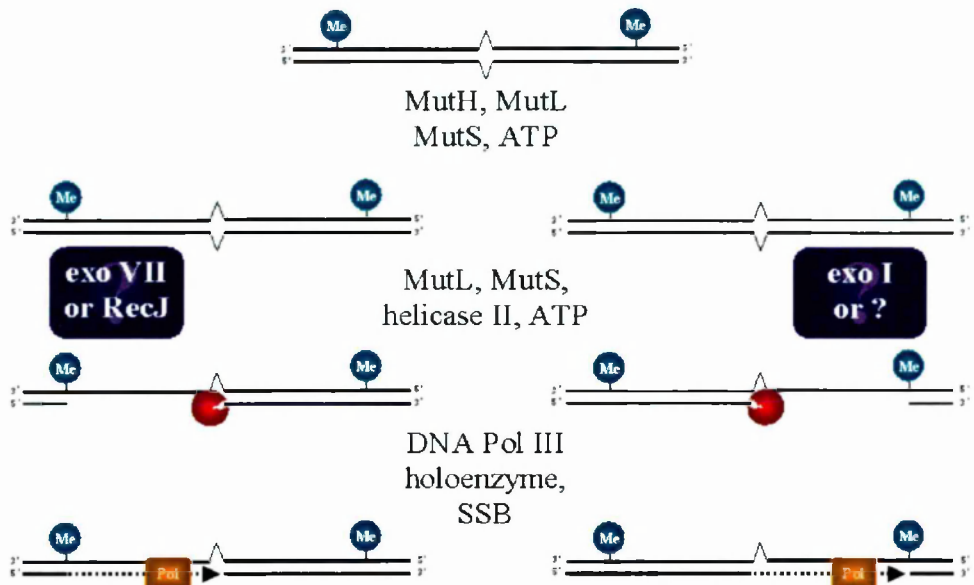
While MutS is capable of recognising mismatched bases and will bind even in the absence of other proteins, nucleotide excision requires the combined activity of the three other proteins; MutH, MutL and UvrD. The endonuclease activity of MutH is very low when present alone, however, in combination with MutS, MutL and a mismatch its activity is stimulated up to 70 fold. Stimulation of the endonuclease activity correlates to the correction efficiency associated with the possible mismatches G:T > G:G > A:C > C:C (Au *et al.*, 1992). This suggests some form of conformation change in the MMR complex stimulated by mismatch geometry. The mismatch repair protein complex appears to be able to track along the DNA until it recognises a GATC site but still retain contact with the mismatched base pair. MutH incises the non-methylated strand at the GATC site, if both strands are unmethylated

incision can occur on either strand. Tracking is bi-directional with respect to the mismatch and proceeds until the nearest GATC site is found either 5' or 3' relative to the mismatch. The bases between the nick and the site of the mismatch are removed, by a 3' to 5' exonuclease if the mismatch resides 3' to the GATC site or by a 5' to 3' exonuclease if it lies 5' to the incision site. The exonucleases thought to be involved in these actions were exonuclease I and another unidentified protein supplying the 3' to 5' exonucleolytic activity with exonuclease VII and RecJ degrading DNA with 5' to 3' polarity Figure 1.21.1. However, recently Harris *et al.* (1998) have constructed a triple Exo I, Exo VII and RecJ mutant and found that its spontaneous mutation rate was unchanged compared to wild type cells. This suggests that these enzymes are either totally redundant *i.e.* other activities can compensate for their absence or that they are not involved in mismatch repair and other exonucleolytic components exist. Excision tracts can be large with the average size being in the region of 1500 bases. Tracts of this size are repaired by Pol III and DNA ligase.

As well as repairing errors introduced by replicative polymerases, mismatch repair is also involved in preventing illegitimate recombination events between diverged sequences (Rayssiguier *et al.*, 1989; Worth *et al.*, 1994; Zahrt and Maloy, 1997). Worth *et al.* (1994) have shown that mismatch repair can dramatically inhibit recombination between sequences differing by as little as 3%. Loss of mismatch repair resulted in up to a 1000-fold increase in recombination frequency when sequence divergence was around 20% (Rayssiguier *et al.*, 1989).

Figure 1.21.1

Methyl-directed mismatch repair (MMR)



Representation of MMR showing recognition of a mismatch followed by cleavage at the nearest GATC site and excision of the unmethylated strand by Exo I, Exo VII or RecJ nucleases, depending on the site of incision either 5' or 3' relative to the mismatch. Recent results would suggest that there are other nucleases that can fully complement the absence of these three enzymes, which are thus not absolutely required for functional MMR. The excision tract is resynthesised, usually by Pol I, to repair the mismatch. MMR only functions on hemi-methylated (indicated by green circles) or fully unmethylated DNA. Since most, newly synthesised DNA is rapidly methylated within minutes of replication, MMR only has a limited time in which to locate and repair mismatches.

Recombination can be still further enhanced by the production of activated RecA protein (Matic *et al.*, 1995) which is thought to increase strand transfer between donor and recipient DNA molecules.

In this thesis a variety of mismatch repair deficiencies have been shown to increase the recovery of deletion mutants in the *trpA* gene of *E.coli*. This follows other work linking defective mismatch repair with instability of DNA sequences (Levinson and Gutman, 1987b) and illegitimate recombination events. The results from this study are discussed in more detail in section 3.1 of this thesis.

1.22 Very short patch repair

E.coli also contains a short patch mismatch repair system typified by excision tracts of 10 base pairs or less. The best-characterised system is very short patch repair (VSP) mismatch correction which removes thymine from G:T mismatches. Sites where 5-methylcytosine occurs have been shown to be hot spots for mutagenesis (Coulondre *et al.*, 1978; Lieb, 1991). Methylation of internal cytosines in the sequence CC(A/T)GG, giving 5-methylcytosine, is performed by the *dcm*-encoded methylase. Deamination of 5-methylcytosine results in thymine rather than the uracil that results from deamination of normal cytosine and results in T:G mismatches. Repair of these specific mismatches is accomplished by the product of the *vsr* gene which is an endonuclease that introduces a nick 5' to the T in T:G mismatches. Removal of T and replacement by C is mediated by DNA polymerase I (Pol I). While VSP repairs T:G mismatches in the CC(A/T)GG context it will also efficiently repair T:G mismatches in a number of other contexts (Lieb and Rehmat, 1995). Thus, there is overlap between VSP and MMR which also efficiently repairs T:G mismatches, it may be that MMR compensates for VSP when the latter's concentration is limited or in sequence contexts less efficiently recognised by VSP repair.

1.23 Nucleotide excision repair

NER is a broad-spectrum repair system that can target a variety of DNA lesions mainly those that are bulky and distort the DNA structure including AP-sites, psoralen adducts, CPD's and thymine glycols (Van Houten, 1990). Unlike more specific damage repair systems that are able to recognise the lesion that is their specific substrate NER has a different damage location mechanism. The currently accepted model is that the UvrA₂B complex tracks along DNA and flexes the duplex testing for an atypical response. Once it finds a lesion the UvrBC component incises the DNA 5' and 3' to the damage. The resulting oligonucleotide is then displaced by UvrD and the single strand gap repaired by Pol I activity. Like *uvrA* and *uvrB*, *uvrD* is damage inducible and is part of the SOS response although there are basal levels of all the proteins involved in NER. For a recent review of NER, see Sancar (1996).

1.24 Damage inducible translesion synthesis

The SOS response is a *recA* dependent damage tolerance and repair system present in *E.coli*. The operon consists of around 16 genes under the control of the LexA repressor protein. The SOS inducing signal, which may be arrested replication forks, single stranded DNA or secondary effects such as altered helicity of the chromosome, leads to activation of RecA protein. This in turn mediates cleavage of the LexA repressor and induction of the genes under its control. While all the proteins induced under the SOS response are involved in the cells recovery from DNA damage and resumption of replication only UmuC and UmuD proteins are

necessary for mutagenesis (Sommer *et al.*, 1993). Strains containing *umuC* or *umuD* mutations are largely non-mutable by a variety of mutagens including UV but are not so sensitive to their lethal effects as are *recA* or *lexA(ind⁻)* mutants. This lack of mutagenesis does not apply to mutagens or events that produce directly mispairing lesions and so do not require SOS to result in a mutation.

The precise function of the UmuC and UmuD proteins remain a mystery but they do increase the ability of DNA polymerase III to bypass non-coding lesions in DNA, a ten fold increase in bypass of a single lesion was observed in an *in vitro* system (Rajagopalan *et al.*, 1992) and *in vivo* (Lawrence *et al.*, 1990; Napolitano *et al.*, 1997). While the two proteins enable *trans*-lesion synthesis, there is no evidence that they interact directly with the polymerase. An alternative is that RecA directs UmuC and UmuD to the site of the lesion and that the proteins will only work on DNA coated with RecA. There is some evidence that activated RecA protein has some affinity for UmuC and activated UmuD proteins (Freitag and McEntee, 1989; Frank *et al.*, 1993) but there is as yet no *in vivo* evidence to support this. Some indicators point towards UmuC and D proteins being involved in restarting DNA replication after it has stalled at the site of a lesion. These proteins, while not normally involved in restarting stalled replication forks, are essential to the process in the presence of certain *recA* alleles (Echols and Goodman, 1991).

SOS mutagenesis is a process that the cell takes some pains to repress until it becomes necessary for survival. This is unsurprising as SOS does increase the mutation rate of the cell, which is something that is unwelcome under most

circumstances. Some recent work has suggested that in situations where *E.coli* is under stress the SOS response may be induced resulting in increased levels of mutagenesis (Taddei *et al.*, 1995; Taddei *et al.*, 1997a). Perhaps amongst its other functions, SOS provides a mechanism for stationary cells to enhance their mutation rate and their ability to adapt to the environment in which they exist.

1.25 Base excision repair

Base excision repair (BER) is a multistep process involving release of the damaged base and leaving behind an AP-site. This is then subject to the attentions of specific AP-endonucleases although some BER enzymes have associated AP-endonuclease activity. Finally, exonucleases process the exposed 5' and 3' termini to leave very small excision tracts in the duplex DNA, this may range from a single base up to about 25-30 bases. The gap is filled by the action of a DNA polymerase and sealed by DNA ligase.

Base excision repair differs from repair processes such as MMR and NER because the enzymes involved are usually much more specific in the type of damage they recognise. *E.coli* contains a large number of such enzymes having specificity ranging from deoxyuracil in U:G mispairs to 8-oxo-dG to thymine glycols. Because BER encompasses such a wide spectrum of repair activities, only a few selected examples will be described.

Uracil is problematic for the cell when it occurs in DNA and can lead to C:G→T:A

transitions if unrepaired. *E.coli* contains a glycosylase that specifically recognises uracil when present in DNA. Uracil DNA *N*-glycosylase is the product of the *ung* gene and can recognise uracil in single or double stranded DNA but not in RNA or RNA/DNA hybrid molecules.

As outlined above, alkylation of DNA bases is also a problem that the cell needs to cope with in order to avoid mutations. *E.coli* possesses two enzymes involved in alkylation repair, 3-methyladenine (3-MeA) DNA glycosylases coded for by the *tag* and *alkA* genes. While the product of *tag* (3-MeA DNA glycosylase I) is fairly specific for removal of 3-MeA and to a lesser extent 3-MeG, 3-MeA DNA glycosylase II the product of *alkA* is unusual in that it has a very broad specificity range. Its substrates include hypoxanthine, 5-methyloxidised thymines, 8-oxo-dG, and ethano or etheno modified purines (Habraken *et al.*, 1991; Bessho *et al.*, 1993; Bjelland *et al.*, 1994; Saparbaev and Laval, 1994). Recently, Berdal *et al.* (1998) have advanced the suggestion that 3-MeA DNA glycosylase II has a general role in removal of DNA lesions and does so by lowering the activation energy for *N*-glycosylic bonds in general with no particular base specificity. Excision efficiency is then a function of the stability of the base attachment to the DNA backbone with modified bases having a reduced sugar bond stability being released more rapidly than normal bases. One of the consequences of this model is that the rate at which normal bases are spontaneously released from DNA would be expected to increase in the presence of this glycosylase. Berdal *et al.*, (1998) have found such an effect, where overexpression of AlkA resulted in an increased spontaneous mutation frequency, with biologically significant release of guanine and other bases from

DNA. This may provide an explanation why, under normal condition, *alkA* is tightly controlled and is induced only when needed, *i.e.* under conditions of alkylation exposure.

A multienzyme repair system is responsible for repairing oxidised guanine residues in DNA. Known as the oxidised guanine repair system or ^oG, it consists of the products of the *mutM*, *mutY* and *mutT* genes (reviewed in Miller, 1996; Fowler and Schaaper, 1997). MutT is a specific nucleoside triphosphate pyrophosphohydrolase, converting 8-oxo-dGTP to 8-oxo-dGMP and pyrophosphate and is responsible for removing the oxidatively damaged triphosphate from the nucleotide pool. While not strictly a DNA repair enzyme its activity will be considered here. 8-oxo-dGTP is a potent mutagen in that it is readily incorporated opposite C or A by Pol III (Maki and Sekiguchi, 1992). Mutants deficient in MutT can have spontaneous mutations rate up to 1000 fold higher than normal (Treffers *et al.*, 1954; Tajiri *et al.*, 1995) and show a unique specificity for A:T→C:G transversions (Yanofsky *et al.*, 1966a; Cox and Yanofsky, 1967).

MutT has also been shown to hydrolyse normal dGTP (Bhatnagar and Bessman, 1988) and it is possible that the enzyme is closely associated with the Pol III replication complex (Bhatnagar *et al.*, 1991). The function of such an arrangement may be to prevent the incorporation of normal guanine in the *syn* rather than *anti* conformation. Such an event could lead to A:G mispairs during replication and these mismatches are poor substrates for MMR.

The other two components of the ^oG system are MutM and MutY, which are DNA glycosylases and prevent reciprocal G:C→T:A transversions by the incorporation of adenine opposite 8-oxo-dG in DNA. MutM has been shown to remove ring-opened purines and 8-oxo-dG, generated *in situ*, from DNA (Chung *et al.*, 1991; Boiteux *et al.*, 1992). In contrast to the 8-oxo-dG_(syn):A_(anti) base pair which is relatively stable and is resistant to attack by MutM, the 8-oxo-dG:C base pair is readily cleaved (Tchou *et al.*, 1991). The initial step in MutM action is the removal of the 8-oxo-dG base to leave an AP-site. MutM also has AP-lyase activity cleaving the sugar phosphate leaving both 3' and 5' phosphoryl groups. The resulting single nucleotide gap is processed to remove the 3' phosphates, for example by exonuclease III, and sealed by DNA polymerase and DNA ligase.

If 8-oxo-dG is not removed prior to replication it has a high probability that adenine will be incorporated opposite it and lead to 8-oxo-dG:A mispairs, as has been stated these are poor substrates for MutM. In this situation, it is the activity of MutY that preferentially removes A when paired with 8-oxo-dG and when paired with normal G (Au *et al.*, 1989; Michaels and Miller, 1992; Tsai-Wu *et al.*, 1992). The AP-site is then subject to excision by an AP-endonuclease and the resulting gap sealed. The 8-oxo-dG in the opposite strand may code for adenine in which case it again becomes the substrate for MutY or for cytosine in which case MutM can excise the 8-oxo-dG lesion. In this way, MutM and MutY co-operate to prevent oxidative lesions in guanine from resulting in heritable mutations (Michaels *et al.*, 1992a; Michaels and Miller, 1992; Tchou and Grollman, 1993). The consequences of 8-oxo-dG

incorporation and the mechanisms that cells deal with the consequences are illustrated in Figure 1.25.1.

Many DNA glycosylases do not contain associated AP-endonuclease activity and leave AP-sites once they have removed the damaged base. These sites are subject to repair by AP-endonucleases whose function is to produce nucleotide gaps repairable by DNA polymerase. *E.coli* has two well-characterised AP-endonucleases, exonuclease III the product of *xthA* gene and endonuclease IV coded for by *nfo*. Exonuclease III has a number of different activities including 3' to 5' exonucleolytic activity on DNA duplex substrate, 3' phosphodiesterase activity, phosphatase activity as well as 5' AP-endonuclease activity. The AP-endonuclease activity may be the major function *in vivo* and could indicate that while other endonucleases can compensate for the absence of exonuclease III in some circumstances they cannot deal with all AP-sites and may become saturated. Exonuclease III may be under the control of the σ^S sigma factor (Sak *et al.*, 1989) suggesting that AP-endonuclease activity could be of increased importance in stationary cells perhaps due to increased levels of damage.

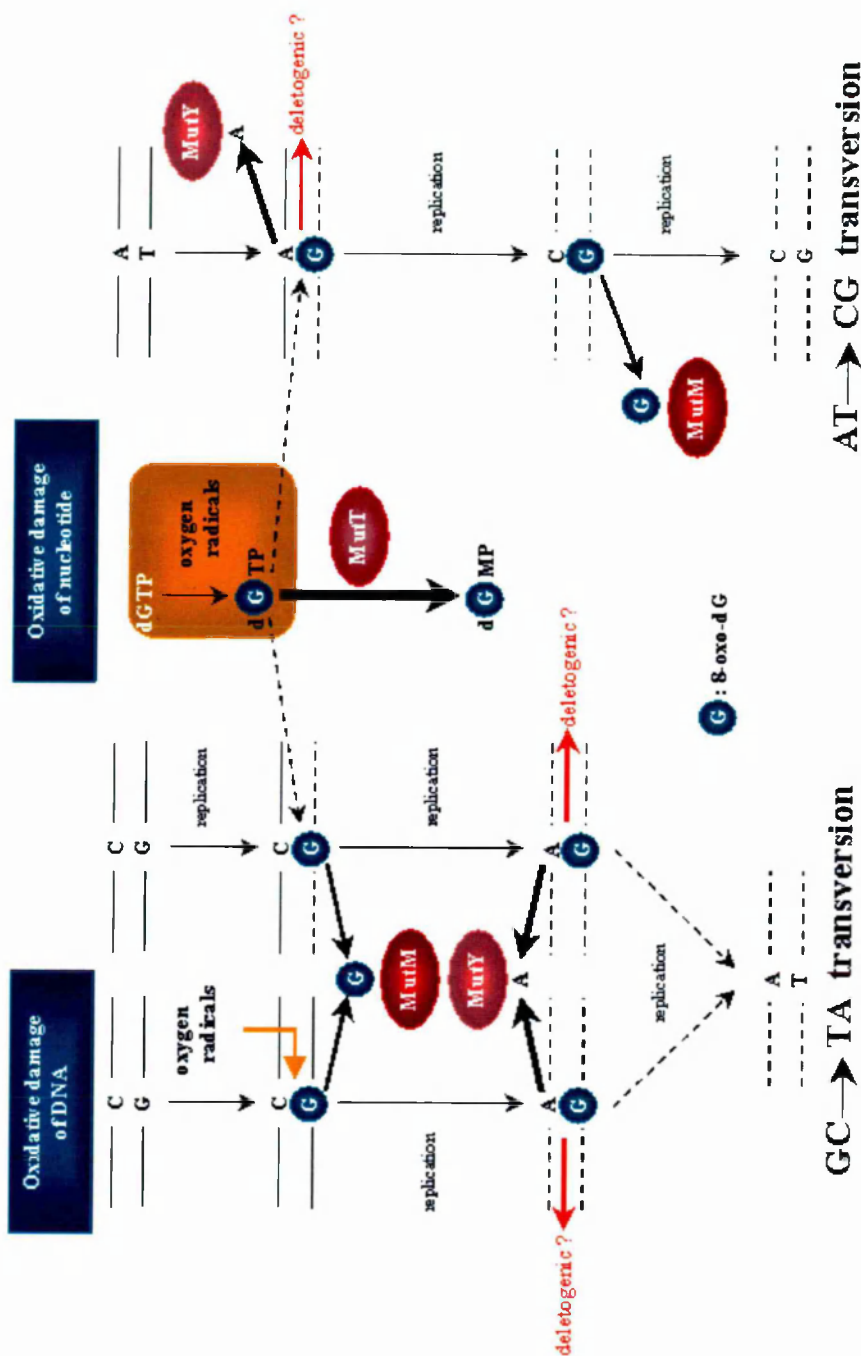
The other enzyme, endonuclease IV, is of minor importance perhaps comprising only 10% of AP-endonuclease activity from cell extracts. Like exonuclease III, endonuclease IV can remove phosphoglycoaldehyde, phosphate and deoxyribose-5-phosphate residues from the 3' termini of duplex DNA enabling them to function as extension termini for polymerases. Under normal circumstances endonuclease IV would appear to be redundant but under conditions of superoxide production,

The ^oG oxidised guanine repair system of *Escherichia coli*

The ^oG repair system deals with oxidative damage to guanine residues. It consists of three enzymes; MutM, MutT and MutY. MutT cleanses the nucleotriphosphate pool by hydrolysing 8-oxo-GTP to 8-oxo-GMP, which can no longer act as a substrate for subsequent conversion back to the triphosphate form. MutM and MutY cope with the effects of 8-oxo-dG when it is incorporated or induced directly in DNA. Incorporation of 8-oxo-dG opposite C provides a substrate for MutM, which removes the damaged G base. If the damage escapes repair prior to replication there is a high probability that A will be incorporated opposite 8-oxo-dG resulting in a mispair. MutY targets A:G mismatches, specifically excising the mispaired adenine, as illustrated this can result in GC→TA transversions. If 8-oxo-dG is incorporated opposite A during replication MutY can recognise the mismatch but excises the template A resulting in a A:T→CG transversion. Subsequent replication may then generate 8-oxo-dG:C pairs which are the substrate for MutM. Based on the relative mutator effects of the three proteins, under normal circumstances MutT will prevent incorporation of most 8-oxo-dG during replication. The MutM and MutY proteins will thus function correctly to reduce mutations from 8-oxo-dG produced directly by oxidative damage of DNA and not themselves act to fix mutations.

Figure 1.25.1

The $^{\circ}\text{G}$ oxidised guanine repair system of *Escherichia coli*



its level is increased by 10-20 fold suggesting its importance in dealing with some forms of oxidative damage.

1.26 Recombination repair

Homologous recombination has been extensively reviewed, for example by West (1992; 1997) and Kowalczykowski *et al.* (1994). There are three main pathways for homologous recombination in *E.coli* and these are usually divided into the RecBCD, RecE and RecF pathways. However, one gene is common to all three pathways and that is the product of the *recA* gene. The RecA protein has a number of functions including cleavage of LexA repressor protein (SOS induction), cleavage of UmuD protein and catalysis of homologous pairing and strand exchange of single and double strand DNA.

DNA recombinational repair is critical to cell survival for example when both DNA strands are damaged (double strand break or cross-linked) the information for accurate repair must come from another homologous DNA molecule *via* recombination. Hence, mutants lacking RecA show extreme sensitivity to DNA damaging agents. It would seem particularly important in the protection against the effects of oxidative damage (Dukan and Touati, 1996) perhaps indicating the propensity of such damage to introduce strand breaks either directly or following processing by a repair enzyme, or to produce lesions that block replication. Mutations in genes such as *xth*, *ung*, *lig*, *polA* and *dam* that cause an increase in the number of DNA strand breaks generally exhibit a hyper-recombinogenic phenotype.

The levels of DNA damage-precipitated breaks and the clear correlation between DNA damage and rates of genetic exchange (Smith and Wang, 1989) strongly suggest that genetic exchanges in bacteria are nearly always associated with DNA damage.

The requirement for strand breaks is clear and is due to the need for single stranded DNA in order for RecA to assemble and initiate pairing. DNA ends can provide entry to exonucleases and helicases to expose single strands for synapse formation. Candidates include the RecBCD enzyme after an encounter with a chi site, RecJ nuclease in conjunction with the RecQ helicase or RecE nuclease to expose a 3' tailed duplex. All these nucleases produce 3' tailed molecules, which are apparently favoured by RecA during synapse formation. Exonuclease I which digests DNA in the 3' to 5' direction is a potent inhibitor of recombination in the absence of RecBC. Since single stranded DNA is rapidly coated by single strand binding protein (SSB), RecA must overcome any inhibitory effect of SSB possibly with the aid of RecO and RecR (Umezumi *et al.*, 1993; Sandler and Clark, 1994) to coat the DNA in a helical filament. The DNA/RecA filament searches for homology by an unknown mechanism and when homology is found the two strands are drawn into alignment. A minimum homology length of 50 base pairs seems to be required for the major RecA mediated recombination pathway to operate (Watt *et al.*, 1985). In duplex/duplex pairings, the result is a four-stranded Holliday junction, which is then resolved by RuvAB and RuvC proteins.

Absence of RecA has noticeably less effect on cell viability than does the absence of

RecBCD, RuvABC or RecG. This implies that double strand breaks are a common occurrence in *E.coli* and have to be repaired, by these protein complexes, for the cell to survive. The apparent dispensability of RecA may be explained if the major double strand lesion encountered by growing cells is a single end formed when a replication fork collapses (Kuzminov *et al.*, 1994; Kuzminov, 1995a; 1995b). Blocked replication forks seem to be particularly involved in deletion formation between long repeat sequences (Louarn *et al.*, 1991; Bierne and Michel, 1994) but may also be involved in promotion of non-homologous illegitimate recombination (Bierne *et al.*, 1997b; Seigneur *et al.*, 1997). In this model, RecBCD-Chi interactions and RecA allow the end to reinvade the intact duplex and restore the replication fork. In the absence of RecA, the broken arm can be degraded allowing reinitiating of replication at *oriC*.

1.3 Starvation associated mutagenesis

The physiology of bacteria in stationary phase is very different from that of logarithmically growing cells (reviewed in Kolter *et al.*, 1993). Clearly, processes that have been elucidated in growing bacteria may behave quite differently in starving cells giving rise to sometimes-unexpected results. Since the work of Luria and Delbruck (1943) later refined by Lederberg and Lederberg (1952), mutants have been thought to arise prior to challenge with a selective agent *i.e.* they were pre-existing mutants that had arisen randomly in the culture. Most investigation into mechanisms and consequences of the processes involved in mutagenesis have been performed on cells that are either actively growing or have very recently stopped

growing *i.e.* in a saturated overnight culture. Measurements of mutation rates are also almost universally made as by Luria and Delbruck themselves using agents that are lethal to unadapted organisms, such as antibiotics or phage resistance. The following section will detail some experiments that cast doubt over these assumptions. It will also look at some of the potential causes and mechanisms behind the observations, which are beginning to illuminate the process of stationary phase mutagenesis.

Luria and Delbruck were fully aware that their system could not reveal any contribution from adaptive responses; they did after all use a lethal selection. Studies conducted by Ryan (1955; 1963) using a non-lethal selection, growing *his* auxotrophs in media lacking histidine, appeared to show that mutations could arise in bacteria that were not actively increasing in number (Ryan, 1963). Other more recent systems have observed that the rate of *araB-lacZ* fusions, caused by excision of Mu, is increased after a period of selection on arabinose-lactose plates or on glucose plates followed by plating to Ara Lac medium (Shapiro, 1984; Maenhaut-Michel and Shapiro, 1994; Foster and Cairns, 1994; Maenhaut-Michel *et al.*, 1997). Activation of Mu excision may be dependent on a physiologically regulated cellular signal network including H-NS which is implicated in gene repression as well as DNA supercoiling and deletion formation (Shapiro, 1993; Gómez-Gómez *et al.*, 1997; Maenhaut-Michel *et al.*, 1997). Reversion of amino acid auxotrophies have also been examined (Hall, 1988; 1990; 1991; Bridges, 1993; 1994) as well as the system that has received most attention, the reversion of a *lacZ* allele carried on an F' episome in the strain FC40 (Cairns *et al.*, 1988; Cairns and Foster, 1991; reviewed by Foster, 1993; 1998). In all cases, mutants arose after incubation on selective plates at a rate

greater than could be accounted for by the observed level of cell division and appeared to be confined to selected genes. The paper by Cairns *et al.* (1988) was controversial in that it suggested "cells may have mechanisms for choosing which mutations will occur". This stimulated research and discussion of the mutagenic processes occurring in starving bacteria variously known as Cairnsian, directed, adaptive, selection-induced or starvation-associated mutagenesis.

Initially, because the only mutants detected were those that resulted in growth, mutagenesis was thought to be directed only at the target gene. Recently this was shown not to be the case; mutations do occur at an increased rate in some other genes and in a variety of replicons other than the F', including plasmids and on the chromosome (Foster, 1997; Torkelson *et al.*, 1997). These findings negate the hypothesis that mutations other than those reverting the selected genes are necessarily transitory (Cairns *et al.*, 1988; Hall, 1990). The mutations resulting in reversion to lactose utilisation have been shown to arise by single base deletions in a run of iterated bases (Foster and Trimarchi, 1994; Rosenberg *et al.*, 1994) and produce a quite different sequence spectrum to mutations arising in growing bacteria.

The reversion of the episomal *lacZ* allele in FC40 requires RecA and RecBCD functions (Cairns and Foster, 1991; Harris *et al.*, 1994) and expression of F' conjugation functions although not actual conjugation itself (Foster and Trimarchi, 1995a; 1995b). Pol III also has a direct role in producing adaptive mutations in this system (Foster *et al.*, 1995; Harris *et al.*, 1997a). While Pol II has been shown to synthesise chromosomal and episomal DNA *in vivo* especially in stationary cells

(Escarceller *et al.*, 1994; Foster *et al.*, 1995; Rangarajan *et al.*, 1997) it does so with relative accuracy. It is noteworthy however that Pol II is part of the SOS regulon and Taddei *et al.* (1995; 1997a) have recently shown that SOS is induced in old colonies on agar plates.

The role of MMR is not entirely clear in the formation of adaptive mutations. The mutation spectrum of episomal *lac* revertants can be reproduced in mismatch deficient cells (Longerich *et al.*, 1995) and is typical of unrepaired polymerase errors. Under normal circumstances single base deletions (or insertions) are corrected by MMR which prevents simple repeat sequence instability (Dohet *et al.*, 1986; Schaaper and Dunn, 1987; Cupples *et al.*, 1990). In stationary cells, the levels of MutS and MutH proteins decrease (Feng *et al.*, 1996) although DNA synthesis levels also decrease to a comparable level? MMR activity may however be modulated as Harris *et al.* (1997b) have found that MutL protein effectively limits mismatch repair in stationary cells. In this way, MMR may be transiently down regulated or depleted in starved cells allowing polymerase errors to persist.

A model incorporating the above observations has been put forward by both Rosenberg *et al.* (1996) and Foster (1998). In brief, a single strand break is produced at *oriT* on the episome during initiation of conjugal replication. On encountering this single-strand nick a replication fork, initiated at one of the episome's vegetative origins, collapses creating a double strand break. Recombination is then initiated by RecBCD producing a single strand that invades homologous sequences allowing replication to resume. Resolution of the resulting Holliday junction towards the

replication fork creates a tract of doubly unmethylated DNA, subject to random repair by MMR if errors are repaired at all. While non-repair or random repair of double strand breaks may account for the appearance of mutations on the episome both in the *lac* allele and adjacent regions (Foster, 1997), it is harder to reconcile this with the observation of Torkelson *et al.* (1997). Their observation of unselected mutations in different replicons implies a *trans*-acting factor.

The observations of Torkelson *et al.* (1997) are more consistent with the original suggestion of Hall (1990), that adaptive mutants arise in a subpopulation of cells that experiences a genome-wide hyper-mutation state. In this model, a subpopulation of cells during selection enters a state of hyper-mutation, producing sequence changes at a high rate. If a cell generated a useful mutation it would exit the state and resume growth. Some of the mutants would also carry unselected mutations, which is exactly what Torkelson *et al.* (1997) and Foster (1997) appear to have found.

Reversion of a *lac* allele on an episome is perhaps a special case of adaptive mutation, involving unique requirements and mechanisms. Other systems have utilised chromosomal genes to look at reversion of amino acid auxotrophies under starvation conditions. Both Hall (1995) and Bridges (1993) have used tryptophan auxotrophs as test systems although different alleles were used in each case. Their results indicate that every system used to measure adaptive mutagenesis should be treated individually. Unlike the FC40 system, both found that their systems did not require functional RecA. However, Bridges (1993; 1995b) found no effect of inactive NER on the number of mutants arising over time whereas Hall (1995) found that

uvrA, *uvrB*, *uvrC* and *uvrD* were all selection-induced specific mutator loci increasing the number of mutants by up to 10,000 fold.

The requirement for, or observation of leaky growth in adaptive mutagenesis has been noted in a number of studies (Bridges, 1994; Jayaraman 1995; Galitski and Roth, 1996; Timms and Bridges, 1998) and may be important for the expression of mutants formed under starvation conditions. This is by no means a universal observation, Foster (1994) did not find any evidence that the number of FC40 bacteria increased during incubation on minimal lactose plates despite the continued appearance of *lac* revertants. Leakiness would have a direct effect on the level of protein and DNA synthesis *in vivo*. Ryan realised early that it was important to measure the rate at which DNA was synthesised in stationary cells to assess whether the level of synthesis was sufficient to account for the number of mutants observed. When protein synthesis was inhibited by chloramphenicol bacteria were able to continue DNA replication for one to two hours (Nakada and Ryan, 1961), but in bacteria that had been starved for histidine or leucine no DNA synthesis was observed (Ryan *et al.*, 1961) using their density labelling technique. Subsequently other studies, including those of Grigg and Stuckey (1966), Couch and Hanawalt (1967) and Tang *et al.* (1979), have shown that DNA synthesis does take place in stationary bacteria. This synthesis was non-conservative and confined to short patches, which is consistent with it being repair synthesis rather than replicational synthesis. Tang *et al.* (1979) showed that most of the synthesis was due to Pol I and was reduced by 90% in a *polA* strain and he estimated that up to 20% of the chromosome was "turned" over in 24 hours. Compared to other systems, 24 hours is

a short time period and there is evidence that this residual DNA synthesis rapidly falls off after the first day or so.

All these studies suffer from the same limitation, which is the use of exogenous label, hence they may all be underestimates of the true level of DNA synthesis in cells. DNA turnover *in vivo* could re-use excised nucleotides as well as breakdown products from RNA. Studies by Bridges (1996a) and Bridges and Ereira (1998), using a *mutT* strain to measure incorporation of oxidatively damaged guanine *in vivo*, have shown that the level of DNA synthesis may be far greater than previously thought (reviewed in Bridges, 1997a). The level is still not high enough to account for all the mutations observed and interestingly unlike the results of Tang *et al.* (1979) does not appear to be *polA* dependent (Bridges and Ereira, 1998). The potential re-use of endogenous nucleotides raises the interesting question does the cell incorporate ribonucleotides into DNA during this starvation-associated synthesis? Pol I has been shown to possess limited reverse transcriptase activity (Ricchetti and Buc, 1993) and could allow replication past template ribonucleotides during DNA turnover in starving cells. However, subsequent replication of these chimeric molecules could also lead to targeted mutations (Thaler *et al.*, 1995).

The *trpE65* and *tyrA14* amino acid auxotrophies used by Bridges (1993; 1994) show starvation-associated mutagenesis. These alleles may respond to endogenous damage perhaps produced by the oxidation of guanine residues to form 8-oxo-dG (Bridges, 1994; 1995a; 1996a; Bridges *et al.*, 1996) and are quite clearly different from the mutants that arise in growing cells (Bridges, 1994). 8-oxo-G is implicated in the

leakiness of some genetic markers due to transcriptional errors. Oxidative damage to guanine can occur in RNA as well as DNA and when damage is to the nucleotide triphosphate precursor pool can lead to misincorporation during transcription **Figure 1.3.1**. Such mistranscription can lead to enhanced production of active gene product and result in some growth of auxotrophs even on starvation plates (Taddei *et al.*, 1997b; Bridges, 1997b), which has implications for the subsequent production of heritable stationary phase mutations. Mistranscription may also result in production of error prone DNA polymerases and other transient mutator molecules. The role of endogenous DNA damage in the process of mutagenesis in stationary phase is considered further by Bridges (1996b)

Could other types of endogenous damage be responsible for the mutations in stationary cells? Strains deficient in the two O⁶-methyltransferases, Ada and Ogt, show increased spontaneous mutation frequencies especially during stationary phase (Rebeck and Samson, 1991; Taverna and Sedgwick, 1996). This suggests the generation of an endogenous mutagen in starving cells which is normally counteracted by increasing levels of Ada upon entry to stationary phase, a process mediated by the stationary phase sigma factor RpoS. Taverna and Sedgwick (1996) consider that it is nitrosation activity catalysed by nitrate reductase that is responsible for the inferred increase in methylation activity.

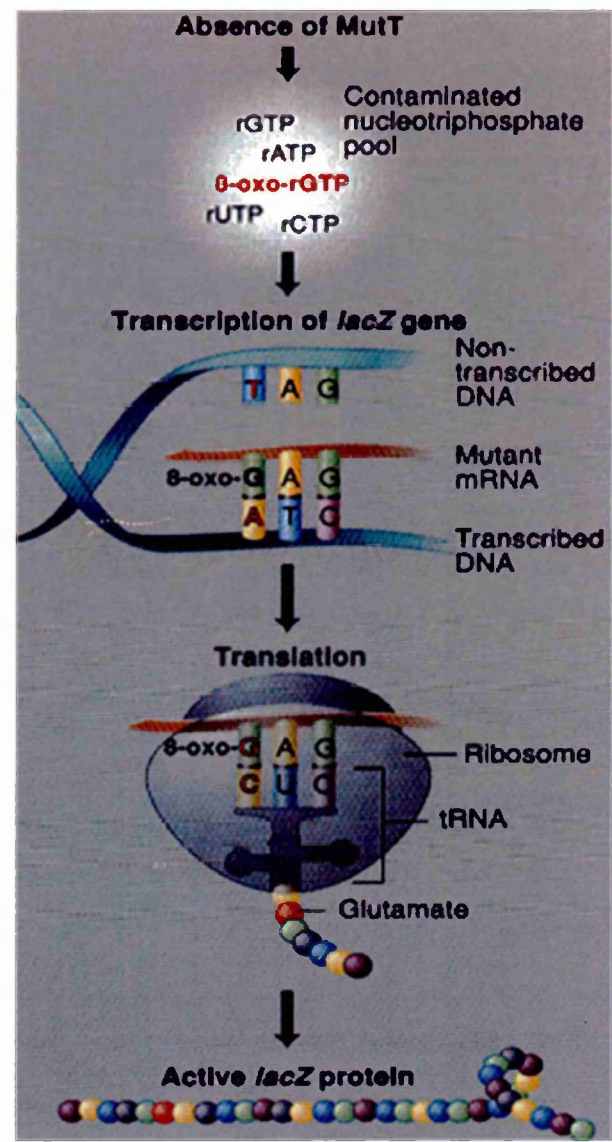
5-methylcytosine is responsible for mutational hotspots in growing *E.coli* through deamination of cytosine producing T:G mispairs. Many, but not all, T:G mispairs are repaired in growing cells by VSP *in vivo*.

Phenotypic leakiness induced by 8-oxo-rGTP

8-oxo-rGTP produced by oxidative damage of the nucleotriphosphate pool is normally cleansed by MutT. In MutT mutants this damaged base accumulates and is occasionally incorporated into RNA opposite template A. The example illustrated involves misincorporation, opposite A of an ATC (amber) triplet, in the *lacZ* coding region. This results in phenotypic correction of the mutation, during translation, leading to a functional *lacZ* product; β -galactosidase. The amber mutation, which normally prevents expression of functional β -galactosidase thus, becomes leaky and allows some growth of the mutant to occur on plates with lactose as a sole carbon source. Figure from Bridges 1997b.

Figure 1.3.1

Phenotypic leakiness induced by 8-oxo-rGTP



Using a back mutation system Lieb and Rehmat (1997) demonstrated that the rate of 5-methylcytosine deamination was not greatly different in growing and starving bacteria but that VSP repair was more efficient under the latter conditions. The increased efficiency of the repair system probable reflects the increased time it has to remove T:G mispairs between replications as compared to actively growing bacteria.

Other forms of damage may occur in starving cells. Part of the cells response to such DNA damage is the induction of the SOS operon. Taddei *et al.* (1995; 1997a) have found that old colonies growing on agar plates show SOS induction, perhaps stimulating DNA repair and recombination functions, a concomitant increase in unselected mutations to rifampicin resistance was also found. Whether this is the same process as that observed in the hyper-mutation of FC40 under selective conditions remains to be seen although both processes are RecA dependent.

Apart from processing of double-strand breaks, implicated in adaptive mutagenesis of an episomal *lac* allele what other mechanisms could account for starvation-associated mutations. The link between transcription and increased mutagenesis has been known for along time (Brock, 1971; Herman and Dworkin, 1971). This is perhaps not surprising, transcription involves separating the DNA duplex producing single stranded DNA, which is more susceptible to damage by chemical mutagens, and spontaneous chemical changes *e.g.* increased depurination or deamination or the production of aberrant secondary structures. Soon after the paper by Cairns *et al.* (1988), Davis (1989) advanced the hypothesis that the apparent "directed" nature of adaptive mutagenesis could be explained by transcriptional bias, an idea later expanded by Wright (1997). The reaction of *E.coli* to starvation for amino acids is called the stringent response which inhibits rRNA, tRNA and cell wall synthesis while activating catabolic and amino acid biosynthetic operons (Cashel *et al.*, 1996). Therefore, systems using amino acid auxotrophies may well result in induction of the operons under selection and do not necessarily only lead to mutations at the selected loci.

Translation is a less accurate process than either DNA replication or transcription, with an error rate of about 10^{-4} /incorporation (Ninio, 1991a; reviewed by Kurland, 1992). When streptomycin is used to alter the translational fidelity of wild type and Sm^R ribosomes a significant increase in mutation frequencies can be demonstrated (Boe, 1992). This may result from the production of a mutator molecule for example inactive ϵ Pol III proof-reading subunit or α subunit with reduced base selectivity. Thus, a few cells in a culture may have a high probability of producing multiple mutations during this transient state *i.e.* prior to degradation of the aberrant protein. Starvation is likely to affect the level of charged tRNA molecules and may affect the ability of the ribosome to correctly read a gene transcript. Some sites have been shown to be inherently less accurately translated in stationary cells than in growing bacteria and may have implications for production of mutator molecules (Barak *et al.*, 1996a; 1996b).

Two sections of this thesis examine the effects of amino acid starvation in *E.coli*. In section 3.1 the contribution of persisting base pair mismatches on deletion formation in the *trpA23* gene is considered. The mutational spectrum obtained in starving cells is different to that obtained in growing cultures and models involving secondary structure or aberrant replication in deletion formation are considered. Section 3.2 looks at a new gene, *tas*, that is required for mutation of the *tyrA14* strain WU3610 to tyrosine independence under conditions of tyrosine starvation. The requirement for leaky growth supplied by *tas* and the possible mutational targets resulting in tyrosine independent mutants are considered.

1.4 Multiple Mutations

There are a number of studies in *E.coli*, yeast and mammalian systems that have found a high proportion (up to 20%) of mutants that contain multiple mutations in genes under selection (Hampsey *et al.*, 1988; Harwood *et al.*, 1991; Timms and Bridges, 1993; discussed by Ninio, 1996). These mutations may have arisen as simultaneous events *e.g.* through the action of an error prone polymerase or by successive events targeted to a specific area of the genome. Ninio (1991b) calculated that a small fraction (about 3×10^{-4}) of an *E.coli* culture could present a transient mutator phenotype and would be responsible for most of the double mutational events. This enhanced mutation may occur over the whole genome or, as seems to be the case in *rpsL* (Timms and Bridges, 1993), is confined to a localised region. Ninio (1996) has advanced the hypothesis that it is repair patches, produced by MMR, that result in linked mutations. The mutations produced would occur within the size of a repair patch *i.e.* within 1-2 kb.

An alternative is to assume that lengths of DNA can become tagged in some way, perhaps during formation or repair of a pre-mutagenic lesion, which focuses subsequent mutagenic events to the same region. One possibility is that bases are chemically modified and inherited in a stochastic fashion. The effects of the targeting would thus become progressively less important in population terms as the tag was diluted, unless a mutation gave rise to a mutant with a substantial selective advantage. This appears analogous to the situation observed in *rpsL* of *E.coli* where about 20% of newly arising Sm^{D} mutants contain an associated second site mutation

in *rpsL* (Timms *et al.*, 1992). The mutation rate for these second sites was calculated to be in the region of 10^{-4} to 10^{-3} /base pair/generation and no evidence of general mutator activity was observed. Established strains did not retain an enhanced mutation rate either generally or at the *rpsL* locus.

Secondary structure may be important in focusing mutations *i.e.* their processing generates mutagenic intermediates. Formation of palindromes may perturb replication leading to complex sequence changes (Glickman and Ripley, 1984; Ripley, 1990) especially if synthesis is initiated from processed secondary structures or MMR repairs mismatches generated during pseudo-palindrome formation.

The possibility that the protein product of a particular gene affects local error rates must also be considered. This has been demonstrated in at least two case for *recA* and *mutS* mutants that increase the mutation rates of their respective genes by up to 10^4 fold (Liu and Tessman, 1990; Liu *et al.*, 1993; Liu *et al.*, 1997). The possibility that *rpsL* is also susceptible to such an effect is considered in section 3.3.

2 General methods

This section describes general methodology and unless otherwise stated applies to all subsections of the work. Specific methods are described in each individual section, as are appropriate strain and plasmid constructions.

Culture methods

Bacteria were routinely grown overnight with shaking in nutrient broth #2 (Oxoid Ltd, Basingstoke, UK) at 37°C. Where appropriate, antibiotics were added to the media at concentrations given in Table 2.1. Bacteria required for starvation experiments were centrifuged, washed twice with 10ml of phage buffer (Boyle and Symonds, 1969), and resuspended in 5ml or 10ml of phage buffer as appropriate. Variable numbers of bacteria were plated according to the requirements of each experiment. Viability determinations, between one and four days, were performed in one of two ways. Either whole plates were washed with 10ml of phage buffer or plugs of agar were taken with a cork borer and resuspended in 10ml of buffer. Appropriate dilutions were plated on to rich media plates with antibiotics if necessary. Minimal agar plates were prepared using the salts solution of Davis and Mingioli (1950) supplemented with 0.4% glucose. Unless otherwise stated media was solidified with 1.5% Difco Bacto agar (Difco Laboratories, West Molesey, UK). Amino acids were added, where appropriate, to a final concentration of 10µg/ml. Antibiotics and amino acids were obtained from Sigma Ltd, Poole, UK.

Genetic manipulation

P1(cir100) transductions were performed as described by Miller (1992). Standard DNA isolation and plasmid manipulations were as described in Sambrook *et al.* (1989).

Table 2.1**Antibiotic concentrations**

Antibiotic	Final concentration $\mu\text{g/ml}$	Abbreviation
ampicillin	100	Ap
chloramphenicol ^a	150	Cm
	12.5	
kanamycin	50	Km
nalidixic acid	4	Nal
novobiocin	500	Novo
rifampicin	100	Rif
streptomycin	1200	Sm
tetracycline	15	Tet

^a Cm resistant plasmids were maintained at the higher concentration, P1(cml) lysogens were selected on the lower concentration of chloramphenicol.

PCR boiling prep

One millilitre of an overnight culture was centrifuged at 14000rpm and, in initial experiments, the pellet washed with 1ml of phosphate buffered saline (PBS). In later experiments 1ml of double distilled water was substituted and was found to work equally well. The pellet was resuspended in 50 μl of water and placed at 99.9°C in a thermal cycler for 10 minutes. The cell debris was removed by centrifugation and the supernatant transferred to a clean tube. Five microlitres of the cleared supernatant was used to seed the appropriate Polymerase Chain Reaction (PCR).

PCR

Reactions were carried out in a total volume of 100µl. Initially the PCR buffer consisted of 50mM KCL, 10mM TRIS-HCL pH 8.4, 1.5mM MgCL₂ and 0.01% gelatin but in later reactions this was replaced by a commercial buffer, PARR (Cambio Ltd, Cambridge, UK), with no apparent effect on amplification efficiency. The four nucleotides were present at 125µM per nucleotide and 100pmol of each primer was added. Either 1u of *Taq* polymerase (GibcoBRL, Paisley, UK) or 0.5u of *Supertaq* (Strattech Scientific Ltd, Luton, UK) was added to each reaction. The mixture was covered with 100µl of light mineral oil (Sigma Ltd, Poole, UK). General PCR conditions were: 5 minutes at 94°C then 30 cycles of one minute at 94°C, one minute at 65°C and one minute at 70°C. A final period of five minutes at 70°C was allowed to ensure full extension of all product fragments. The amplified DNA was removed from under the oil layer to a clean tube and the product cleaned, in early experiments with the GeneClean II kit (Bio 101, La Jolla, California) and latterly with the Wizard DNA Cleanup kit (Promega (UK) Ltd, Southampton, UK) according to the manufacturer's instructions. The cleaned product was resuspended in 40µl of water.

Dynabead capture of amplified PCR product

If the PCR product was required for sequencing, one of the PCR primers was modified with a 5' biotin attachment. This enabled the PCR product to be extracted from solution utilising the high affinity streptavidin-biotin linkage (Hultman *et al.*,

1989). Twenty microlitres of Dynabeads-M-280 Streptavidin (DynaL (UK) Ltd, Wirral, UK) were washed twice with 20µl of TWS (0.17% (w/v) Triton X-100, 100mM NaCl, 10mM TRIS-HCL pH 7.5, 1mM EDTA) and finally resuspended in 40µl of TWS. The resuspended Dynabeads were added to the cleaned PCR product and the mixture incubated at room temperature on a rotating shaker for 30 minutes. The Dynabeads and bound DNA were separated using a magnetic particle separator (MPS) and resuspended in 40µl of 200mM NaOH, the mixture was incubated at room temperature for five minutes. The denaturation step, with NaOH, was repeated before a final wash with 40µl of TWS and one with water, each time separating the Dynabeads and DNA using the MPS. Finally, the pellet was resuspended in 7µl of water in preparation for sequencing.

DNA sequencing

Sequencing was invariably performed on single stranded DNA. This was produced either from a PCR product, by Dynabead attachment, or from a phagemid using standard molecular biological techniques. The single stranded DNA was annealed to 5ng of the appropriate primer at 65°C for five minutes followed by 30 minutes at 37°C. The primed DNA was sequenced using the Sequenase V2.0 kit (United States Biochemical, Cleveland, Ohio), extension and termination were carried out according to the manufacturer's protocol. The samples were heated to 85°C immediately prior to loading onto a 6% polyacrylamide gel and run for various lengths of time. The gel was fixed, dried and exposed to X-ray film, usually overnight or longer depending on signal intensity.

3.1 Deletions in the *trpA* gene of *Escherichia coli*

The *trpA23* strain IC3126 and its *mutY* derivative IC3742 were used to examine the contribution of the base analogue 8-oxo-7,8 dihydro-2'-deoxyguanine (8-oxo-dG) to mutations arising in starving bacteria. Using the phenotypic characterisation, developed by Allen and Yanofsky (1963) and Yanofsky *et al* (1966b), mutants arising in growing cultures of IC3742 were inferred to show a high rate of G:C→T:A transversions at the site of the *trpA23* mutation (Urios *et al.*, 1994; Bridges *et al.*, 1996). These mutations resulted in an amino acid substitution giving a functional tryptophan synthase α subunit. However, mutations arising in bacteria starved for tryptophan could not be classified cleanly using the phenotypic methods alone, there were conflicts between the phenotypic observations and the predicted sequence changes expected to have been induced by 8-oxo-dG:A mispairing. In an attempt to resolve this apparent discrepancy, mutants arising during starvation were isolated and sequenced. The sequencing study revealed that as well as the expected G→T transversions there were a high proportion of mutants that contained small in-frame deletions that restored activity to the *trpA* gene.

3.11 Methods

Bacterial strains and plasmids

Bacterial strains are shown in Table 3.11.1. P1 phage transductions were performed as previously described. Strain constructions were verified according to their mutator

Table 3.11.1

Bacterial strains

Strain	Relevant genotype	Source or derivation
B34T	<i>uvrA</i> , <i>zjb1</i> ::Tn10	Laboratory stock
DE1824	<i>umuDC</i> ⁺ , <i>fadR613</i> ::Tn10	R. Woodgate
DL733	Δ (<i>sbcDC</i> :: <i>Km</i> ^R)	D.R.F. Leach
IC3126	<i>trpA23</i> , <i>uvrA155</i> , Δ (<i>umuDC</i>):: <i>cat</i> ^R	M. Blanco
IC3742	as IC3126 but <i>mutY68</i> :: <i>Km</i> ^R	M. Blanco
MT1	<i>rpoS</i> ::Tn10	F. Taddei
RW202	Δ (<i>srIR-recA</i>)306::Tn10	R. Woodgate
WU3610 _(old)	<i>uvrA</i> ⁺	E.M. Witkin
WU3610 <i>mutH</i>	<i>mutH471</i> ::Tn5	R. Bockrath
WU3610 <i>mutL</i>	<i>mutL</i> ::Tn10	R. Bockrath
WU3610 <i>mutS</i>	<i>mutS201</i> :: <i>Km</i> ^R	R. Bockrath
CM1330	as IC3126 but <i>mutL</i> ::Tn10	this work P1 (WU3610 <i>mutL</i>) × IC3126
CM1331	as IC3742 but <i>mutL</i> ::Tn10	this work P1 (WU3610 <i>mutL</i>) × IC3742

Table 3.11.1

Bacterial strains

Strain	Relevant genotype	Source or derivation
CM1343	as IC3742 but $\Delta(srIR-recA)306::Tn10$	this work P1 (RW202) \times IC3742
CM1348	as WU3610 _(old) but $zjb1::Tn10$	this work P1 (B34T) \times WU3610 _(old)
CM1349	as IC3742 but $uvrA^+$, $zjb1::Tn10$	this work P1 (CM1348) \times IC3742
CM1359	as IC3126 but $mutS201::Km^R$	this work P1 (WU3610 $mutS$) \times IC3126
CM1362	as IC3126 but $mutH471::Tn5$	this work P1 (WU3610 $mutH$) \times IC3126
CM1366	as IC3126 but $rpoS::Tn10$	this work P1 (MT1) \times IC3126
CM1367	as IC3742 but $rpoS::Tn10$	this work P1 (MT1) \times IC3742
CM1370	as IC3126 but $uvrA^+$, $zjb1::Tn10$	this work P1 (CM1348) \times IC3126
CM1371	as CM1359 but $uvrA^+$, $zjb1::Tn10$	this work P1 (CM1348) \times CM1359
CM1372	as CM1362 but $uvrA^+$, $zjb1::Tn10$	this work P1 (CM1348) \times CM1362
CM1406	as IC3126 but $\Delta(sbcDC::Km^R)$	this work P1 (DL733) \times IC3126
CM1407	as CM1406 but $mutL::Tn10$	this work P1 (WU3610 $mutL$) \times CM1406
CM1414	as IC3742 but $umuDC^+$	this work P1 (DE1824) \times IC3742

phenotypes for *mutY*, *mutH*, *mutL* and *mutS*. About 10^7 bacteria were plated to minimal agar plates containing limited tryptophan ($1\mu\text{g/ml}$) and the number of Trp^+ colonies arising was compared to the parent strain. Increased resistance to UV irradiation for *uvrA*⁺, excision proficient bacteria were able to survive a dose of 10 Jm^{-2} UV, which was lethal to excision deficient strains. Increased mutagenesis upon exposure to UV for *umuDC*⁺, irradiation with 2 Jm^{-2} UV resulted in approximately a 60% increase in the number of Trp^+ mutants arising in *umuDC*⁺ strains. Finally by examination of hydrogen peroxidase activity, in old colonies, for *rpoS* transductants (Mulvey *et al.*, 1988). The ΔsbcDC allele, although having the kanamycin resistance cassette, does not have an easily verified and independently selectable phenotype. In this case, PCR was used to verify the presence of transduced ΔsbcDC alleles into the recipient strains. PCR conditions were as previously described with 30 amplification cycles. Reactions contained 100 pmoles of primers *sbcDCAU* (5'CGCCGTGCGTGACATTTATA3') and *sbcDCAL* (5'TTGGTTACGCGCCCGATTCT3') to amplify a 930 base pair fragment across the deletion in *sbcDC*. Wild type cells produced a PCR product while *sbcDC* deletion strains gave no product. Antibiotics were added where appropriate, concentrations were as previously described.

Starvation experiments

Cultures were spun, washed, and resuspended in a final volume of 10ml of phage buffer. One hundred microlitres of washed culture (about 10^8 cells) were plated, for mutator strains the cultures were diluted 10 fold and 100 μl plated (about 10^7 cells), on minimal agar plates containing minimal salts and glucose. The plates were

incubated at 37°C for up to 21 days and the number of colonies counted each day. Viability determinations were performed over four days using the whole plate wash off technique and suitable dilutions plated to L-agar plates.

Trp⁺ mutant isolation

Trp⁺ mutants, from growing bacteria, were isolated according to the plating method of (Newcombe 1948), overnight cultures were washed as above and 10⁷ bacteria were plated to minimal agar plates containing 0.25µg/ml of tryptophan. The plates were incubated at 37°C and mutant colonies arising between days five to eight were isolated for sequencing. Mutants arising between days five to nine on starvation plates, minimal agar plates without additional tryptophan, were picked off at day nine and patched to fresh minimal agar plates and grown at 37°C. In later experiments, only slower growing mutants (taking more than 36 hours to form a visible patch on minimal plates) were selected for sequencing.

Phenotypic characterisation of Trp⁺ mutants

Revertants of *trpA23* can be characterised by correlating their sensitivity to 5-methyl-DL-tryptophan (5-MT) with indole-glycerol phosphate (IGP) accumulation. To test 5-MT inhibition isolated Trp⁺ mutants were patched to minimal agar containing 0.5µg/ml tryptophan and 0.1µg/ml of 5-MT and incubated for 24 hours at 37°C. To test IGP accumulation, a liquid minimal culture of each mutant was centrifuged and 1ml of the cleared supernatant was mixed with 1.5ml of ferric chloride reagent (1ml

of 0.5M FeCl₃, 50ml of water and 30ml of concentrated H₂SO₄). Cultures of partial revertants give a pink colour due to IGP accumulation, full revertants give no colour. The inferred base pair substitutions associated with each phenotype, according to the scheme of Yanofsky *et al.* (1966b), are shown in Table 3.11.2.

Table 3.11.2

Characteristics of *trpA23* revertants

Revertant class ^a	Sensitivity to 5-MT ^b	IGP accumulation	Inferred base pair substitution
I FR	R	-	A:T→G:C A:T→C:G A:T→T:A
II PR	R/S	Faint	G:C→C:G
III PR	S	+	G:C→T:A

^a FR, full revertant; PR, partial revertant.

^b R, resistant; S, sensitive; R/S slightly sensitive.

Sequencing of Trp⁺ mutants

Overnight cultures, of Trp⁺ mutants, were inoculated in nutrient broth, and grown at 37°C with shaking. Boiling preps were prepared as previously described. Five microlitres of cleared lysate was used in PCR to amplify a 452 base pair fragment of the *trpA* gene, encompassing the site of the *trpA23* mutation at codon 211. The PCR contained 100pmole of primers TRPA1 (5'XGCCCGCCAAATGCCGATGACGA3') and

TRPA2 (5'CGGGGTAAGCGAAACGGTAAAAAGATA3'), TRPA1 is biotinylated (X) at the 5' end. PCR conditions were as described previously and 30 amplification cycles were performed. PCR products were cleaned using the Wizard DNA cleanup kit (Promega (UK) Ltd, Southampton, UK) according to the manufacturers instructions. The cleaned PCR product was attached to Dynabeads-M280 Streptavidin (Dyna (UK) Ltd, Wirral, UK) and denatured as previously described. Sequencing was carried out using primer TRPA3 (5'AGCATTCTCTGGCTCAT3') as previously described.

3.12 Results

In an attempt to verify the predicted sequence changes resulting in tryptophan independent mutants, arising during a starvation experiment, 20 mutants isolated from the *trpA23 mutY* strain IC3742 were sequenced. Phenotypic data and the corresponding inferred and actual mutation spectrum for the region of the *trpA* gene adjacent to the site of the *trpA23* mutation are shown in Table 3.12.1. Where there was disparity between the observed phenotypes, for 5-MT resistance and IGP accumulation, the inferred base pair change (G:C→C:G) was the best available option amongst those listed in Table 3.11.2 to fit the data. In this experiment, sequencing data spanned a window of about 130 base pairs encompassing the *trpA23* site. Changes outside this region, leading to a Trp⁺ phenotype, would not have been seen and are denoted as "*trpA23* sequence". Based on the phenotypic data alone most of the 20 mutants characterised were inferred to have a G:C→C:G change in the *trpA* gene the sequence spectrum, however, shows a completely different pattern.

Table 3.12.1

Phenotypic and sequence comparison in a series of
Trp⁺ mutants of IC3742

5-MT resistance	IGP accumulation	Inferred sequence	Observed sequence ^a
+	Faint	G:C→C:G	G632T
+	Faint	G:C→C:G	<i>trpA23</i> sequence
-	+++	G:C→T:A	Δ657(8)-668(9)
+	Faint	G:C→C:G	G632T
+	Faint	G:C→C:G	G632T
+	++	G:C→C:G	G632T
+	+++	G:C→C:G	Δ644(6)-646(8)
+	++	G:C→C:G	G632T
+	++	G:C→C:G	G632T
-	++	G:C→T:A	<i>trpA23</i> sequence
+	+++	G:C→C:G	Δ650(2)-667(9)
-	+++	G:C→T:A	Δ644(6)-646(8)
+	++	G:C→C:G	<i>trpA23</i> sequence
+	Faint	G:C→C:G	G632T
-	+++	G:C→T:A	Δ646-654
-	+++	G:C→T:A	Δ660(1)-662(3)
+	++	G:C→C:G	G632T
+	Faint	G:C→C:G	G632T
+	Faint	G:C→C:G	G632T
+	Faint	G:C→C:G	G632T

^a Where the deletion is flanked by direct repeats the possible range of the deletion is indicated in brackets. Sequence changes refer to the coding strand of *trpA*, with the first base of the ATG initiation codon taken as position 1.

As predicted in a *mutY* strain most of the sequence changes found were G:C632T:A transversions resulting from miscoding events opposite 8-oxo-dG. In this first experiment the transversions were exclusively at the *trpA23* site resulting in a mutation changing arginine to isoleucine, which was already known to give a functional revertant (Yanofsky *et al.*, 1966b).

Amongst the mutants, there was also a high proportion of more complex changes in *trpA* involving the formation of small in-frame deletions near to the site of the *trpA23* mutation. In this first experiment, six deletions all resulted in an in-frame fusion presumably giving a partially active tryptophan synthase protein. There were also three mutants that showed no changes within the region sequenced. It was apparent during isolation of the mutants, for sequencing, that those found to contain deletions grew substantially slower than the majority of mutants containing point mutations, taking 2-3 days to grow on a minimal agar plate compared to 1 day. There were a number of point mutations that also showed slow growth specifically C:G692A:T and C:G698A:T, however growth rate was a useful preliminary screen to eliminate most point mutations prior to sequencing. There is the possibility that the occasional fast growing deletion would have been missed. However based on our observations, this class would not account for a large proportion of the population.

A second set of 20 mutants was isolated from IC3742, isolated in the same way as the first experiment except that growth rate was used to screen out fast growing mutants. The results are shown, along with experiment one for comparison, in Table 3.12.2. In the second experiment we also found a single in-frame deletion out of 10

Table 3.12.2

Nature of deletions found among Trp⁺ revertants of the *trpA23* strain IC3126 and its *mutY* derivative IC3742 arising during tryptophan starvation

Strain	Experiment	Sequencing window	Revertants screened	Bases deleted ^a	Amino acids deleted	Times found
IC3126	1	564-703	65	644(6)-646(8)	216	6
				672-677	225-226 I(ATT)224 I(ATA) ^b	1
	2	564-719	84	673(4)-678(9)	225-226	2
				644(6)-646(8)	216	4
				672-677	225-226 I(ATT)224 I(ATA) ^b	1
IC3742	1	550-682	20	644(6)-646(8)	216	2
				646-654	216-218	1
				650(2)-667(9)	218-223	1
				657(8)-668(9)	220-223	1
				660(1)-662(3)	221	1
	2	565-715	20	664(8)-675(9)	222(3)-225(6)	1

^a Numbers in parentheses refer to deletions flanked by direct repeats and the possible end points of the deletions.

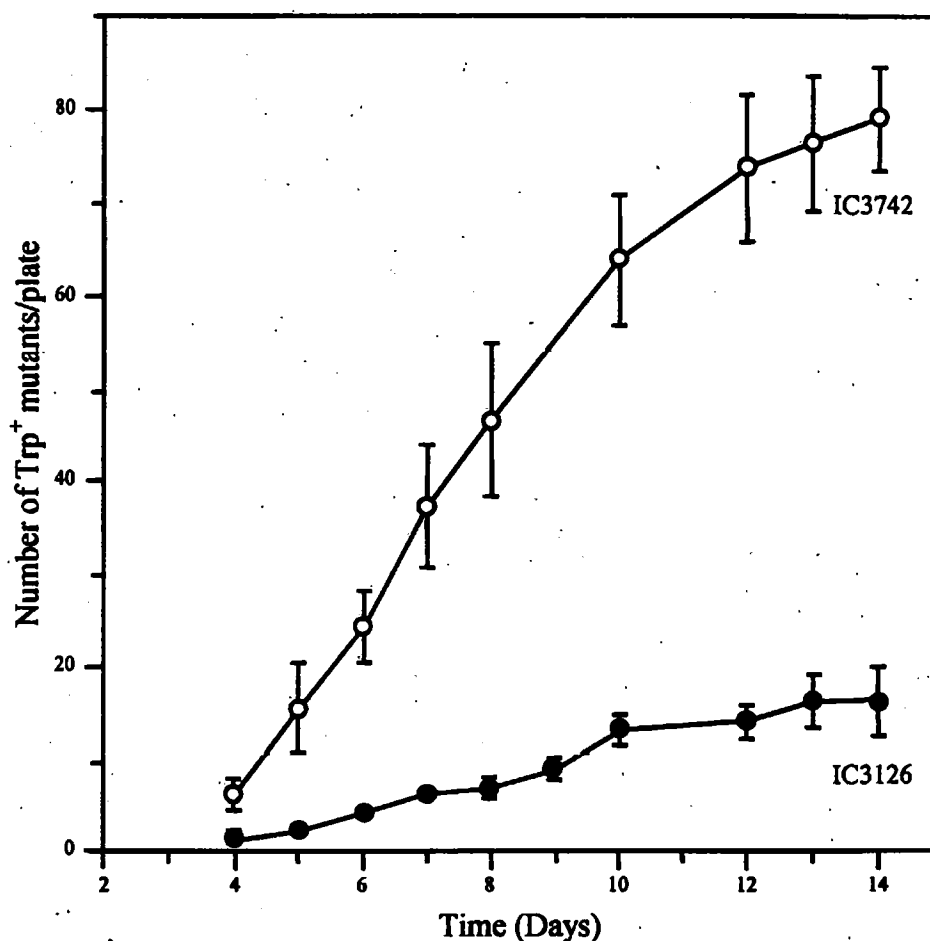
^b Also involves a change of codon for the indicated amino acid.

mutants sequenced, the remainder were point mutations. Interestingly $7/10$ of the mutants, identified as slow growing, contained a G:C→T:A transversion at the site of the *trpA23* mutation. This revertant, with an arginine to isoleucine change at codon 211, was usually discarded during the growth screen as it was relatively fast growing. Their appearance in this and in other sequenced slow growers may be accounted for either i) because the growth screen was subjective and tended to veer on the cautious side, picking excess mutants rather than risk missing true slow growers, or ii) the mismatch repair defective strains produced additional base substitutions and deletions so functional revertants of *trpA23* might also have contained other mutations reducing their growth rate.

As a control, the formation of deletions was examined in the *mutY*⁺ parent of IC3742 under starvation conditions. One hundred and forty nine mutants of IC3126, from two experiments, were characterised according to their growth rate and 18 of them were found to be slow growing. Sequencing revealed that 14 of the mutants carried deletions while three showed no change in the region sequenced and one was a point mutation already known to give a slow growing phenotype Table 3.12.2. The deletions from both experiments were predominantly of a single type, 10 out of the 14 having an identical mutation, flanked by 2 base pair direct repeats one of which was lost during the mutation process. This particular deletion is the predominant type found in all the experiments although the variety of deletions found in mismatch repair defective strains is greater than that found in the parent strain. The rate of appearance of tryptophan independent (Trp⁺) mutants in strains IC3742 and IC3126 is shown in Figure 3.12.1.

Figure 3.12.1

Starvation-associated mutation in IC3126 and its *mutY* derivative IC3742



Starvation-associated mutation experiment with the *trpA23* strain IC3126 (●) and its *mutY* derivative IC3742 (○). Points are the mean of three independent experiments.

The numbers of Trp⁺ mutants arising in IC3126 is between 10-15% of those arising in IC3742, despite both strains showing considerable background lawn growth after 14 days. Deletions in IC3742 account for ($7/40$) 17.5% of the total number of mutants arising on starvation plates, the proportion arising in IC3126 is ($14/149$) or 9.4%.

Given that the total number of mutants arising in IC3126 is no more than 15% of those arising in IC3742 the number of deletion mutants arising in IC3126 is 1.4% i.e. $((^{9.4}/_{100}) \times 15)$ of the total number of mutants arising in IC3742 under identical conditions. Since deletions account for 17.5% of mutants in IC3742 greater than 90% of the deletions we see must be due to the presence of the *mutY* mutation.

The two strains IC3126 and IC3742 also carry two markers that are involved in general DNA repair and in mutagenesis. While functional UvrA, UmuC and UmuD proteins are clearly not essential in deletion formation it may be their absence that is allowing the deletogenic pathway to operate. We constructed a *uvrA*⁺ (CM1349) and a *umuDC*⁺ (CM1414) derivative of IC3742 to examine the effects of functional nucleotide excision repair (NER) and error prone repair. Sixty four mutants of CM1349, from a single experiment, were screened and 22 were found to be slow growing. Sequencing revealed that there were two deletions present, the remainder being point mutations with a single mutant where no change was discernible in the sequenced window. Screening of CM1414 gave 48 slow growers out of 66 mutants isolated of these, six were deletions five were outside the sequenced region and the remainder were point mutations. The results for CM1349 and CM1414 are shown in Table 3.12.3.

When the number of mutants arising over time on minimal agar plates was followed, there was very little difference in the numbers or rate of appearance between IC3742 and its *uvrA*⁺ derivative CM1349. The *uvrA*⁺ derivative of IC3126 (CM1370) is

Table 3.12.3

Nature of deletions found among Trp⁺ revertants of the *trpA23* strains CM1343 (*mutY*, $\Delta recA$), CM1349 (*mutY*, *uvrA*⁺) and CM1414 (*mutY*, *umuDC*⁺) arising during tryptophan starvation

Strain	Experiment	Sequencing window	Revertants screened	Bases deleted ^a	Amino acids deleted	Times found
CM1343	1	530-678	40	644(6)-646(8)	216	2
		565-710		646(8)-663(5)	216-221	1
	2	565-711	20	646-654	216-218	1
CM1349	1	565-702	64	644(6)-646(8)	216	1
				646-654	216-218	1
CM1414	1	565-697	66	644(6)-464(8)	216	2
				646-654	216-218	1
				646(7)-657(8)	216-219	2
				651-659	218-220 P(CCG)217 P(CCA) ^b	1

^a Numbers in parentheses refer to deletions flanked by direct repeats and the possible end points of the deletions.

^b Also involves a change of codon for the indicated amino acid.

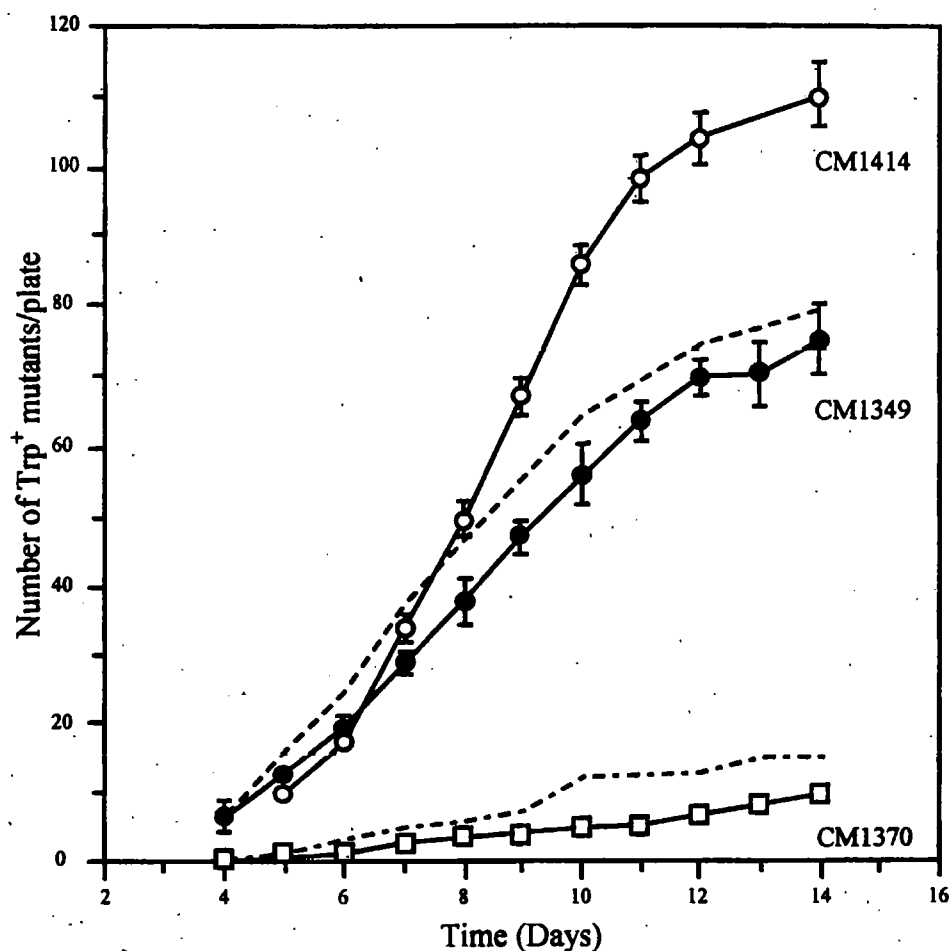
included for reference; its rate of mutant appearance over time was again similar to IC3126. The *mutY umuDC*⁺ strain CM1414 did show a consistent increase in the number of mutants that arose during starvation. The results for strains CM1349, CM1370 and CM1414 are shown in Figure 3.12.2 the profiles for the *uvrA*⁺ strains can be compared to those for the parental strains from Figure 3.12.1.

We constructed a $\Delta recA$ derivative of IC3742 to look at the effect of RecA protein on the formation of deletions. Sixty mutants from two separate experiments were screened and the resulting 11 slow growers consisted of four deletions Table 3.12.3, three unchanged sequences and four point mutations. When the *recA* deletion strain, CM1343, was incubated under tryptophan starvation conditions Trp⁺ mutants arose at a slower rate than IC3742 Figure 3.12.3. When viability of the two strains was examined over the first 24 hours, by plating 1×10^6 bacteria on minimal plates, it was found that the parent strain increased by a factor of 36 while the *recA* deletion strain only increased by a factor of 6 (data supplied by B.A.Bridges). Both of these strains produced equally heavy lawns when incubated over the period of a week. The difference in viability was presumed to be due to lethal sectoring occurring in the $\Delta recA$ strain and is probably the explanation for the lower rate of appearance of Trp⁺ mutants. Again RecA is not essential for the formation of deletions but because of the uncertainty over inviability, it cannot be stated that RecA has no effect on the rate of their formation. Specifically we cannot say whether RecA affects the processing of deletions with and without terminal direct repeats. Both types were present among the four deletions recovered from the $\Delta recA$ strain but the sample is far too small to base a conclusion there on.

Figure 3.12.2

Starvation-associated mutation in the *uvrA*⁺ (CM1349, CM1370)

and *umuDC*⁺ (CM1414) derivatives of IC3126 and IC3742

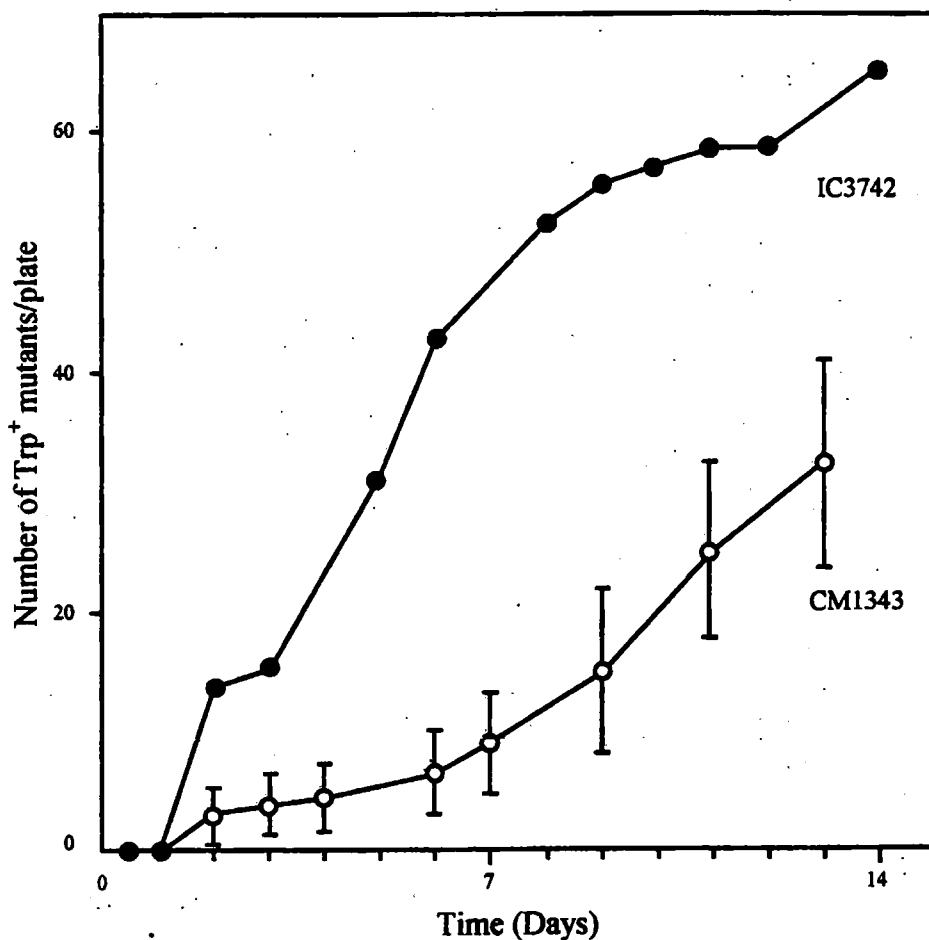


Starvation-associated mutation experiment with the *uvrA*⁺ derivatives of IC3126 (CM1370, □) and IC3742 (CM1349, ●) and the *umuDC*⁺ derivative of IC3742 (CM1414, ○). Points are the mean of three independent experiments. Dotted lines are taken from Figure 3.12.1 for comparison with strains; IC3126 - - - - - and its *mutY* derivative IC3742 - - - - -.

Figure 3.12.3

Starvation-associated mutation in the *mutY* strain IC3742 and its

***ΔrecA* derivative CM1343**



Starvation-associated mutation experiment with the *ΔrecA* derivative (CM1343, ○) of the *trpA23 mutY* strain IC3742 (●) (3×10^8 bacteria per plate). Points for CM1343 are the mean of three independent experiments. Figure from Bridges and Timms, 1997.

The *mutY* gene in *E. coli* codes for an adenine glycosylase, specifically recognising and removing adenine when it is mispaired with guanine or 8-oxo-deoxyguanine (Au *et al.*, 1988; 1989; Michaels *et al.*, 1992a). 8-oxo-dG has similar pairing affinity with

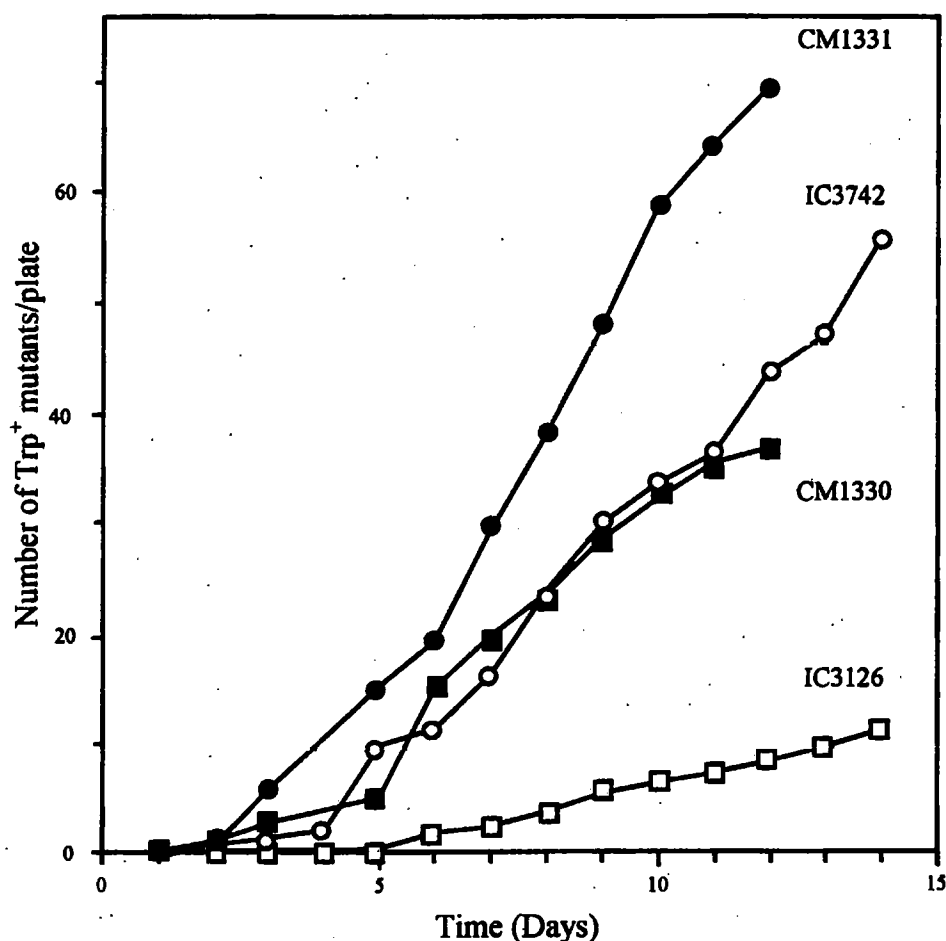
cytosine or with adenine, the inactive *mutY* in IC3742 would thus result in an increase in the number of 8-oxo-dG:A mismatches persisting in DNA. These would ultimately result in G:C→T:A transversions upon DNA replication (Nghiem *et al.*, 1988). One hypothesis is that these mismatches in some way might be related to the high proportion of deletions we see amongst the slow growing mutants.

The major mismatch correction pathway in *E.coli* is methyl-directed mismatch repair consisting of the gene products of the *mutH*, *mutL* and *mutS* genes. A *mutY mutL* double mutant (CM1331) was constructed to see whether methyl-directed mismatch repair was involved in the formation of deletions. Among 40 mutants of CM1331, from two experiments, 10 slow growing mutants were sequenced. Two contained deletions or 5% of the total number screened, showing that the MutL protein is not essential to deletion formation in *mutY* bacteria. The numbers of mutants arising over time under starvation conditions is indicated in Figure 3.12.4, approximately double the number of mutants arise in CM1331 compared to the *mutY* or *mutL* (CM1330) single mutant strains (data supplied by B.A.Bridges). It would appear that the pathways involving *mutY* and *mutL* are working independently, with their effects being cumulative on the number of Trp⁺ mutants arising in starving bacteria.

If *mutY* were inducing deletions by way of 8-oxo-dG:A mismatches, normal mismatches might also be expected to increase the deletion rate. A series of strains, based on IC3126, was constructed which were deficient in one of the three main methyl-directed mismatch repair proteins MutH, MutL or MutS.

Figure 3.12.4

Starvation-associated mutation in the *mutY*, *mutL* and *mutY mutL*
derivatives of the *trpA23* strain IC3126



Starvation-associated mutation experiment with the *mutL* derivatives of the *trpA23* strain IC3126 (CM1330, ■) and the *trpA23 mutY* strain IC3742 (CM1331, ●). Points are the mean of two experiments with 10^7 bacteria per plate. Figure from Bridges and Timms, 1997.

In the case of CM1330 (*mutL* strain) two experiments were performed and 11 slow growing mutants were sequenced. For CM1359 (*mutS*) and CM1362 (*mutH*) tryptophan independent mutants were isolated and screened, for growth rate, from a

single experiment. The sequencing results for the mismatch deficient strains are shown in Table 3.12.4. All three strains showed a 9.3-15% proportion of deletions amongst the mutants screened also showing a concomitant increase in the number of slow growing mutants arising over time on minimal plates without tryptophan Figure 3.12.5. As with the *mutY* strain, *uvrA*⁺ derivatives were made of the *mutS* strain (CM1371) and *mutH* (CM1372). The NER proficient phenotype had no effect on the number or rate of Trp⁺ revertants arising over time on starvation plates Figure 3.12.6. If the level of deletions in the wild type strain is again taken to be 1.4% of the number in IC3742, the proportion of deletions attributed to the mismatch correction defects are 91% ($(15-1.4)/15 \times 100$) for *mutL*, 85% ($(9.3-1.4)/9.3 \times 100$) for *mutS* and 86% ($(10.2-1.4)/10.2 \times 100$) for *mutH*.

No small in-frame deletions were found in mutants isolated from growing bacteria rather than bacteria that were starved, for an extensive period, of tryptophan. Sixty mutants of IC3742 were examined, isolated using the method of Newcombe (1948) where bacteria are plated on minimal agar plates with limited amounts of the required amino acid. The bacteria grow and form a lawn and normally revertants arising during this growth form colonies in two to three days. Because the deletion mutants were so slow growing mutants appearing between days five to eight were isolated to allow deletion mutants time to express and form colonies. Only one mutant was found that was slow growing and sequencing showed the mutation to lie outside of the sequenced window.

Table 3.12.4

Nature of deletions found among Trp⁺ revertants of the *trpA23* strains CM1330 (*mutL*), CM1331 (*mutL*, *mutY*) CM1359 (*mutS*) and CM1362 (*mutH*) arising during tryptophan starvation

Strain	Experiment	Sequencing window	Revertants screened	Bases deleted ^a	Amino acids deleted	Times found
CM1330	1	554-680	20	624(7)-632(5)	209-211	1
				646(7)-657(8)	216-219	1
				646(8)-663(5)	216-221	1
	2	565-715	20	644(6)-646(8)	216	1
				646-654	216-218	1
CM1331	1	554-680	20	651-659	218-220 P(CCG)217 P(CCA) ^b	1
				646-654	216-218	1
				646(8)-663(5)	216-221	1
	2	565-711	20	none found		

Table 3.12.4

Nature of deletions found among Trp⁺ revertants of the *trpA23* strains CM1330 (*mutL*), CM1331 (*mutL*, *mutY*) CM1359 (*mutS*) and CM1362 (*mutH*) arising during tryptophan starvation

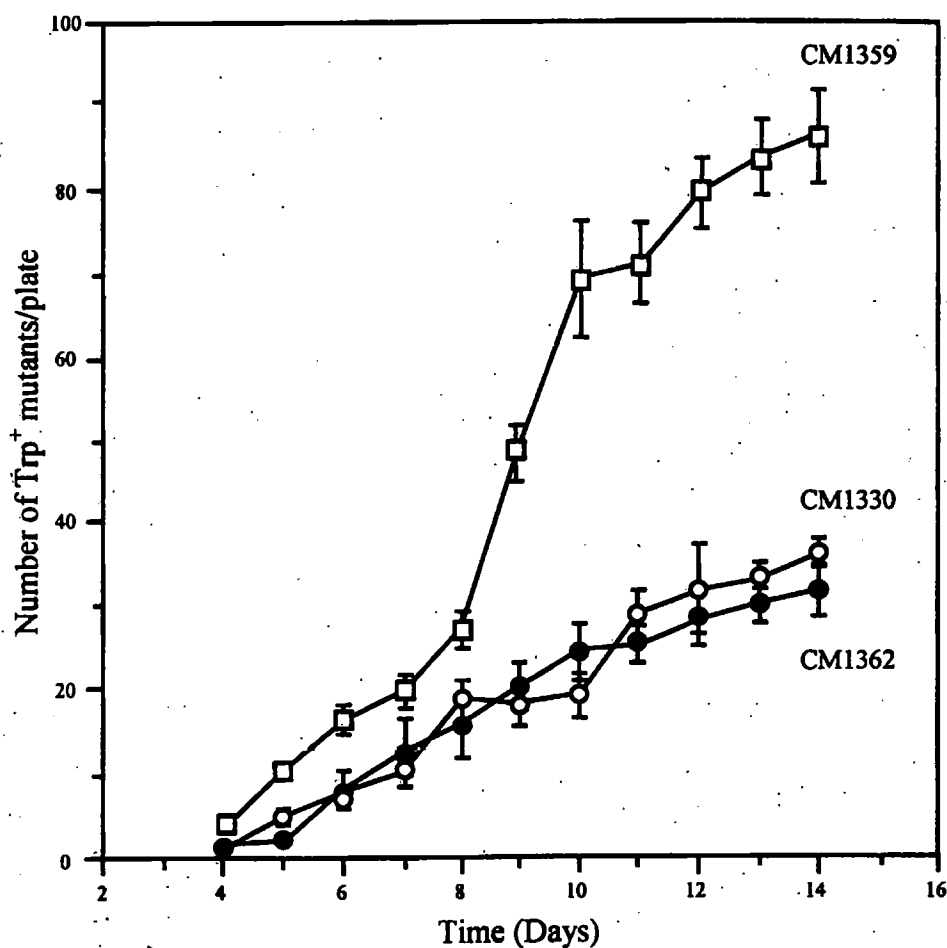
Strain	Experiment	Sequencing window	Revertants screened	Bases deleted ^a	Amino acids deleted	Times found
CM1359	1	570-718	86	644(6)-464(8)	216	4
				646-654	216-218	1
				646(7)-657(8)	216-219	1
				657-659	220 Q(CAG)219 Q(CAA) ^b	1
				658(9)-666(7)	220-222	1
CM1362	1	565-711	88	643-645	215	1
				644(6)-646(8)	216	5
				646(8)-663(5)	216-221	1
				666(9)-680(3)	224-228 A(GCG)223 A(GCT) ^b	2

^a Numbers in parentheses refer to deletions flanked by direct repeats and the possible end points of the deletions.

^b Also involves a change of codon for the indicated amino acid.

Figure 3.12.5

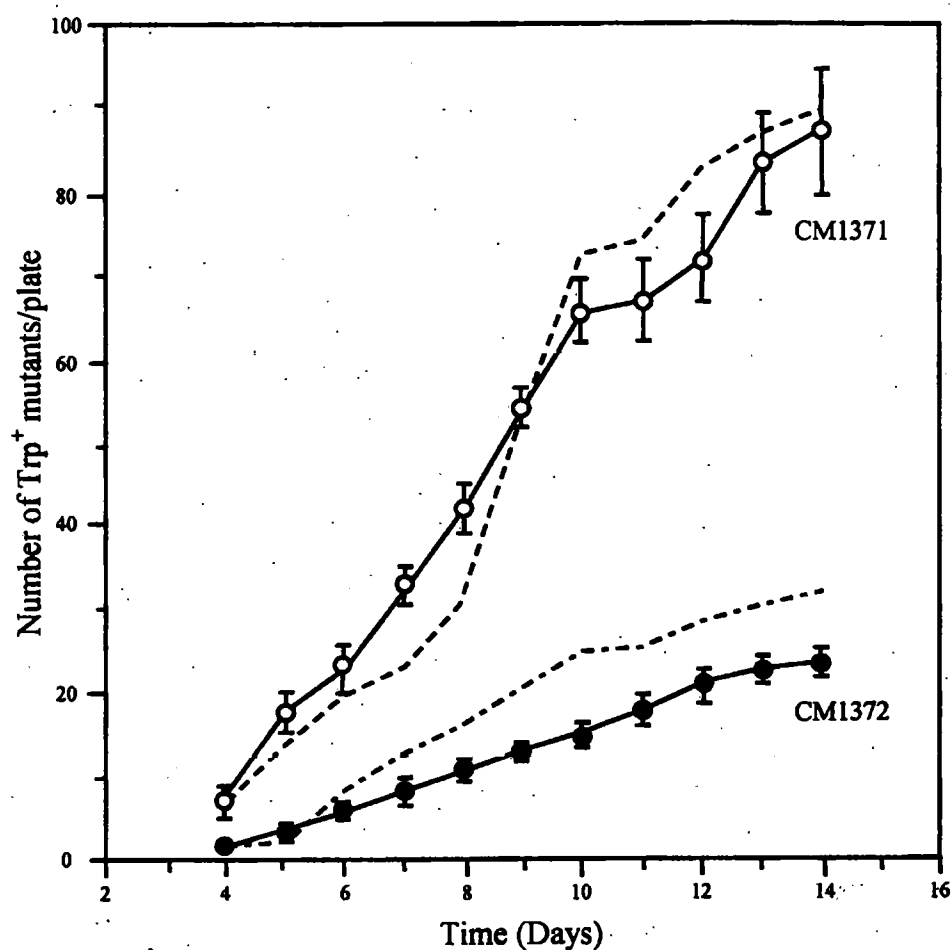
Starvation-associated mutation in MMR defective derivatives of IC3126



Appearance of tryptophan-independent mutants with time of incubation on minimal plates for *E. coli* IC3126 MMR defective derivatives carrying *mutH* (CM1362, ●), *mutL* (CM1330, ○) and *mutS* (CM1359, □). 10^7 bacteria were plated per plate, points are the means of at least three experiments.

Figure 3.12.6

Starvation-associated mutation in excision repair proficient derivatives of *mutS* strain CM1359 (CM1371) and the *mutH* strain CM1362 (CM1372)



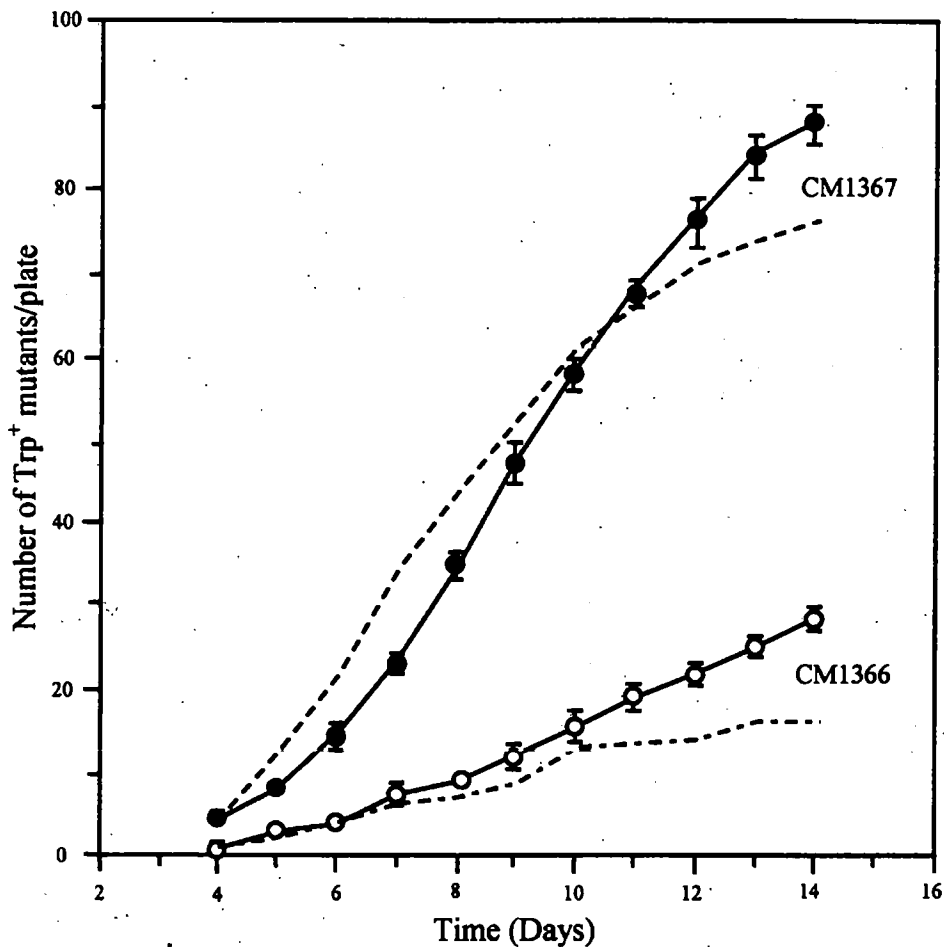
Appearance of tryptophan-independent mutants with time of incubation on minimal plates for *E. coli* IC3126 derivatives carrying *mutH* (CM1372, ●) and *mutS* (CM1371, ○) made excision repair proficient by transduction of a *uvrA*⁺ allele. (10⁷ bacteria were plated per plate, points are the means of at least three experiments). Dotted lines are taken from Figure 3.12.5 for comparison; CM1359, - - - - and CM1362, - - - - .

When combined with other results (B.A Bridges, personal communication) it leads to the conclusion that 8-oxo-dG:A mispairs do not give rise to deletions in growing bacteria within the detection limits of our method *i.e.* they comprise less than 1% of the prototrophs that arise. This suggested that, at the very least, deletion formation was a far more common phenomenon in starving cells than in growing bacteria.

The stationary phase sigma factor σ^S , encoded by the gene *rpoS*, is responsible for the regulation of a large number of genes involved in survival and adaptation of bacteria to life in stationary phase (Kolter *et al.*, 1993; Zambrano *et al.*, 1993; Eisenstark *et al.*, 1996; Hengge-Aronis, 1996a; 1996b; Zambrano and Kolter, 1996). σ^S has been shown to be an absolute requirement for the formation of Mu-mediated fusion events, producing Lac-Ara⁺ mutants, during prolonged starvation (Gómez-Gómez *et al.*, 1997). A number of σ^S regulated genes are involved in the protection of bacteria from the effects of oxidative damage. One particular gene *dpsA*, an abundant non-specific DNA-binding protein, has been shown to be involved in protection of DNA against the effects of oxidative damage (Almirón *et al.*, 1992; Martinez and Kolter, 1997). Strains deficient in Dps have also been shown to exhibit an increased level of G:C→T:A mutations when stationary cultures were challenged with H₂O₂ (Martinez and Kolter, 1997) and the protein has been shown to possess slight catalase activity in a *Synechoccus* species (Marjorette *et al.*, 1995). Because the *trpA23* system was susceptible to this type of mutational event, a *rpoS*::Tn10 derivative of the strains IC3126 (CM1366) and IC3742 (CM1367) were constructed to examine the number and rate of appearance of slow growing mutants. Results are indicated in Figure 3.12.7, they show that there is little difference between either

Figure 3.12.7

Starvation-associated mutation in *rpoS* derivatives of IC3126 and IC3742



Starvation-associated mutation experiment with *rpoS*::Tn10 derivatives of IC3126 (CM1366, ○) and IC3742 (CM1367, ●). Points are the means of at least three experiments. Dotted lines are taken from Figure 3.12.1 for comparison with strains; IC3126 ---- and IC3742-----.

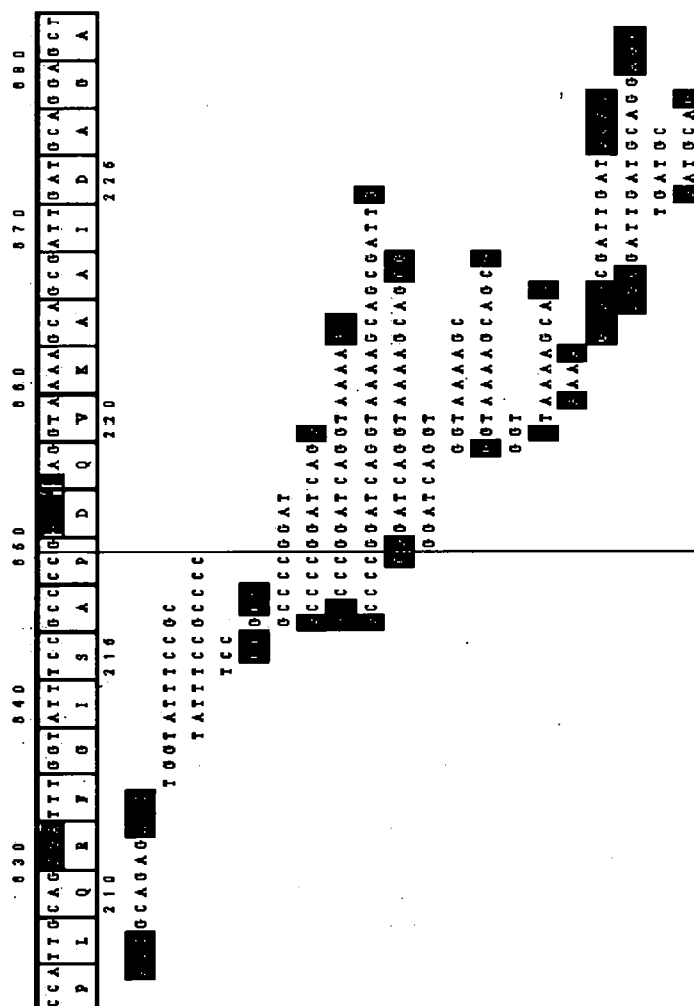
strain with or without functional σ^S . It would seem that in the *trpA23* system σ^S and the genes it controls have no effect on mutations leading to Trp⁺ revertants. This is surprising, in view of the number of genes controlled by σ^S involved in protection

against oxidative damage. However, it is in agreement with other results where no effect of σ^S could be demonstrated in a different starvation mutation system based on reversion of an ochre tryptophan auxotrophy (Bridges and Timms, 1998).

Our sequencing primers were originally designed to look at the specific region immediately adjacent to the site of the *trpA23* mutation. All the deletions we identified during our screening of Trp^+ mutants had termini that lay within our sequenced region lying between nucleotides 624-627 and 680-683. We only observe small in-frame deletions in our assay, presumably because deletion of more than a few amino acids (our longest observed deletion is the equivalent of nine amino acids) would inactivate the protein. The reading frame must also be preserved to give a functional protein, out of frame deletions at this point in the gene (about 57% into the coding sequence) would almost certainly give a non-functional product. The compiled deletion spectrum from all our strains is shown in **Figure 3.12.8**. Base substitution mutations are also included, but they do not constitute a complete spectrum of possible reversion mutations at this locus.

Where deletions have direct repeats at their ends these are indicated, terminal repeats varied from one to four nucleotides and during the deletion process, one of the repeats was invariably lost. Of the deletions sequenced, 61 had terminal repeats of one or more bases and 22 had no direct repeats at their termini. A different pathway, not involving repeat structures, may have formed deletions without repeated termini or the structures involved in their formation are not immediately adjacent to the deleted region.

Deletions in the *trpA* gene of *Escherichia coli*



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Because of the apparent localisation of the deletions, a mechanism was sought that might act as a target to this region of the DNA.

There is a deletion pathway, in phage λ , that operates via the *sbcC* and *sbcD* gene products and is known to process stem-loop structures occurring in DNA (Gibson *et al.*, 1992; Leach, 1996). When the DNA sequence adjacent to the *trpA23* site was examined, it was found that there were two potential pseudo-hairpin structures that might serve to target deletions to this region. A *mutL*, Δ *sbcDC* derivative of IC3126 (CM1407) was constructed. In three experiments a total of 284 Trp⁺ mutants were screened and the 53 slow growers found were sequenced, out of these 25 contained deletions. Sequencing results are presented in Table 3.12.5. 8.8% of the mutants in CM1407 contained a deletion in *trpA*, this is not significantly different from the proportion of deletions found in the other mismatch repair deficient strains and from the 15% present in the *mutL* single mutant. We therefore concluded that Δ *sbcDC* had no effect on deletion formation in our system neither did it affect the number of mutants arising during starvation experiments compared to CM1330 Figure 3.12.9 and to CM1406, the *mutL*⁺ Δ *sbcDC* control strain.

Table 3.12.5

Nature of deletions found among Trp⁺ revertants of the *trpA23* strain CM1407 (*mutL*, Δ *sbcDC*)

Strain	Experiment	Sequencing window	Revertants screened	Bases deleted ^a	Amino acids deleted	Times found
CM1407	1	565-712	71	624(7)-632(5)	209-211	1
				636-647	213-216 F(TTT)212 F(TTC) ^b	1
				639-650	214-217 G(GGT)213 G(GGG) ^b	1
				644(6)-646(8)	216	1
				646-654	216-218	2
				646(8)-663(5)	216-221	2
				646(7)-672(3)	216-224	1
				657-659	220 Q(CAG)219 Q(CAA) ^b	1
				657-665	220-222 Q(CAG)219 Q(CAA) ^b	1
				644(6)-646(8)	216	2
CM1407	2	565-700	104	646-654	216-218	1
				646(7)-657(8)	216-219	2

Table 3.12.5

Nature of deletions found among Trp⁺ revertants of the *trpA23* strain CM1407 (*mutL*, $\Delta sbcDC$)

Strain	Experiment	Sequencing window	Revertants screened	Bases deleted ^a	Amino acids deleted	Times found
				646(8)-663(5)	216-221	1
				646(7)-672(3)	216-224	1
3		565-698	109	644(6)-646(8)	216	3
				646-654	216-218	1
				646(7)-657(8)	216-219	1
				657-659	220 Q(CAG)219 Q(CAA) ^b	1
				660(1)-662(3)	221	1

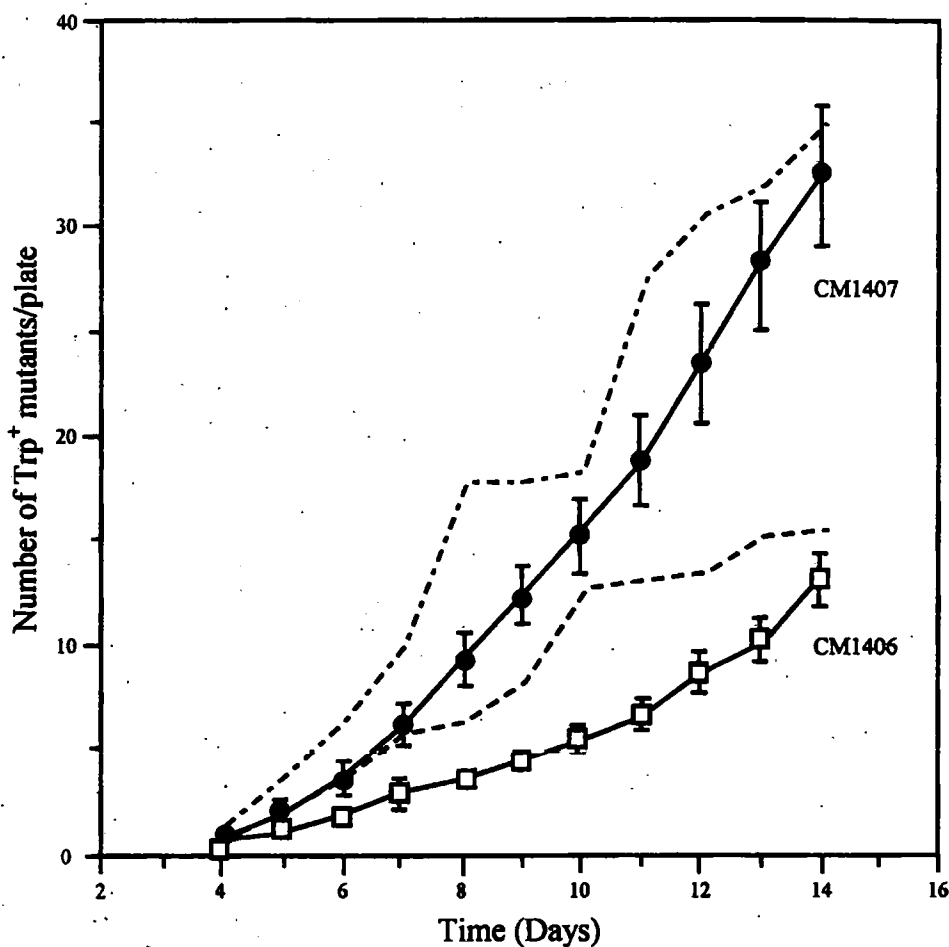
^a Numbers in parentheses refer to deletions flanked by direct repeats and the possible end points of the deletions.

^b Also involves a change of codon for the indicated amino acid.

Figure 3.12.9

Starvation-associated mutation in $\Delta sbcDC$ and $\Delta sbcDC mutL$ derivatives

Of the *trpA23* strain IC3126



Starvation-associated mutation experiment with a $\Delta sbcDC$ derivative of IC3126 (CM1406, ○) and a *mutL* derivative (CM1407, ●). Points are the means of at least three experiments. Dotted lines are taken from Figure 3.12.1 for comparison with strain IC3126 ---- and Figure 3.12.5 for comparison with the *mutL* derivative (CM1330) -.-.-.

3.13 Discussion

There have been a number of reports linking the oxidatively damaged base analogue 8-oxo-dG with mutagenesis in starving bacteria. Strains deficient in MutY or MutT, two enzymes involved in the removal of oxidatively damaged guanine and together with MutM comprising the ^oG repair system, show an increase in the number of mutants arising over time when they are starved for an essential amino acid (Bridges, 1995a; 1996a; Bridges *et al.*, 1996). The exception, MutM, does show a large effect on starvation associated mutagenesis when combined with either of the other two deficiencies. Slow growing Trp⁺ revertants were previously observed to arise, along with an elevated level of faster growing G:C→T:A transversions, when a *mutY trpA23* strain, IC3742, was incubated on minimal agar plates lacking tryptophan (Bridges *et al.*, 1996). The present study began as a characterisation of these slow growing Trp⁺ revertants using a phenotypic assay developed by Allen and Yanofsky (1963) and subsequently by sequencing the region of the *trpA* gene immediately adjacent to the *trpA23* mutation.

In the first experiment with IC3742, phenotypic classification was used, looking at sensitivity of the mutants to 5-methyl-tryptophan (5-MT) and the accumulation of the synthesis intermediate indole-glycerol-phosphate (IGP). This classification scheme makes it possible to infer the base pair substitutions that lead to the Trp⁺ phenotype (Yanofsky *et al.*, 1966b). On the basis of the phenotypic results most of the mutants from the first experiment were classified as G:C→C:G transversions. This was surprising as the *mutY* strain was expected to yield an overwhelming majority of

G:C→T:A transversions. It had previously been shown that, in growing bacteria deficient in MutY, 70-95% of the phenotypic revertants were inferred to contain G:C→T:A transversions at the site of the *trpA23* mutation (Urios *et al.*, 1994). This particular base pair substitution, at nucleotide 632, results in a change from arginine (AGA) at codon 211 (inactive tryptophan synthase α subunit) to isoleucine (ATA) producing a functional protein (Allen and Yanofsky, 1963; Yanofsky *et al.*, 1966b).

Sequencing results have been presented showing that most of the base changes classified as G:C→C:G, on the basis of their phenotype, were in fact G:C→T:A substitutions at nucleotide 632. However there were also six deletions in the sequenced region as well as three mutants that appeared to be unchanged from the *trpA23* sequence.

In most of the mutant isolations a small proportion of slow growing revertants was picked up that did not show a sequence change within the region examined. It was assumed that these mutants were either intragenic or extragenic suppressor mutations. These could range from mutations in ribosomal genes (affecting translational fidelity) to mutations and modifications of tRNA resulting in altered anticodon recognition specificities and in light of our results may also include deletions at other points in the *trpA* gene. For a review of the range of suppression mechanisms operating in bacteria, with particular emphasis on the *trpA* gene, see Murgola (1985). This disparity between the phenotypic and sequencing data, in IC3742, is a pertinent reminder that a large range of mutational mechanisms operate in bacteria, not all of which are understood. It is now clear that it is risky to base

conclusions purely on phenotypic characteristics in this system. There is ample evidence that spontaneous mutational processes occurring in stressed or starving bacteria are different to those that occur in growing bacteria (Cairns and Foster, 1991; Prival and Cebula, 1992; Bridges, 1993; Foster and Trimarchi, 1994; Mackay *et al.*, 1994; Rosenberg *et al.*, 1994; Taddei *et al.*, 1995; Maenhaut-Michel *et al.*, 1997). Our results would appear to extend these observations with the inclusion of a major deletion pathway operating in our system during starvation.

The strains we used in our study were all characterised by a deficiency in a mismatch repair system. MutY and the methyl-directed mismatch repair (MMR) system both operate to remove mismatched base pairs, their inactivation leads to the persistence of such mismatches in DNA. Both of these mismatch repair systems have different repair specificities and as demonstrated in the results section the effect of each deficiency is cumulative on the number of mutants that arise during starvation in this system. In isolation, both repair deficiencies are virtually indistinguishable in the number or rate of mutants that arise over time. In the slow growing Trp⁺ mutants sequenced, we found at least three sites that respond to G:C→T:A transversions in *trpA* to give a functional revertant (G:C632T:A, G:C692T:A and G:C698T:A). These three mutations are all events that could be produced by defects in MutY mediated mismatch repair and resulting from a misincorporation of adenine opposite 8-oxo-dG. Tajiri *et al.* (1995) produced an estimate of the number of 8-oxo-dG molecules in the chromosome of wild type cells, this was approximately 6 molecules per chromosome. Assuming that 8-oxo-dG has similar propensity to pair with adenine as with cytosine during replication (Shibutani *et al.*, 1991; Moriya *et al.*, 1991) then

there could be at least three 8-oxo-dG:A mismatches per chromosome assuming no repair. In cells where all the error correction pathways are operating normally the rate of replicative errors is about 10^{-9} to 10^{-10} /base pair/replication cycle (Drake, 1991a; 1991b; Schaaper, 1993), the effect of abolishing mismatch repair is an approximately 100 to 1000-fold increase in observed errors. The resulting error rate of around 10^{-7} , in a *mutH*, *mutL* or *mutS* strain, translates to about one mismatch for every two chromosomes replicated (4.7M base pairs/chromosome). Since the *trpA23* allele can revert by a variety of base substitutions, including both transitions and transversions, all else being equal there would have to be at least 18 sites ((number of 8-oxo-dG:A mismatches per chromosome/number of MMR susceptible mismatches per chromosome) \times number of sites responsive to G:A mismatches)) susceptible to MMR and that can cause a Trp⁺ revertant to give parity in mutant generation. Combining the sequencing results obtained in this study with those of Yanofsky *et al.* (1966b) there are at least 10 such sites, eight of which were shown to have a slow growing phenotype. Of course, the above takes no account of the range of deletion mutants recovered in the slow growing revertants or of the revertant mutations lying outside the region sequenced. Because MMR does not repair most G:C \rightarrow T:A transversions (Dohet *et al.*, 1985; Schaaper *et al.*, 1989) and MutY is specific for such events there is no overlap between the repair spectra, thus there is an additive effect of removing both repair systems.

In growing bacteria deficiency in mismatch repair results in a large mutator effect, with an increase in the number of base pair substitution mutations as subsequent rounds of replication fix the unrepaired mismatched base (Nevers and Spatch, 1975;

Lu *et al.*, 1983; Pukkila *et al.*, 1983). However, when we looked in starving bacteria, as well as the expected base pair substitutions, we also found a large proportion of small in-frame deletions in *trpA*. We failed to detect any deletions in spontaneous Trp⁺ mutants produced in growing cultures, we concluded they must comprise less than 1% of the mutant population, compared to between 5-17.5% in mismatch repair deficient strains starved for tryptophan.

The *trpA23* system would only appear to be able to detect small in-frame deletions, the largest deletion seen is only 27 base pairs or 9 amino acids and all occur in one region of the *trpA* gene, larger deletions would presumably inactivate the protein as would out of frame deletions or frame shifts. It is not unreasonable to assume that there are perhaps twice as many out of frame deletions that are never seen, as in-frame deletions. The unknown proportion of deletions extending outside the defined region known to give functional revertants should also be considered. The DNA sequence where we find our deletions spans bases 624 to 683 of the *trpA* gene some 60 base pairs, out of which, 30 are G:C base pairs and potentially responsive to 8-oxo-dG:A mispairs. In the *mutY* strain IC3742, 17.5% of the slow growing mutants are small in-frame deletions and 75% carried transversions. If it is assumed i) there are twice as many out of frame deletions that are not detected, as in-frame deletions that are detected, ii) that the deletions originated from mismatches within the deleted sequence and iii) the average probability of transversions at each of these 30 G:C base pairs is the same as that for transversion at the *trpA23* site then, in a *mutY* strain, 52.5% (3×17.5) of events in this region would consist of deletions. The proportion for the other mismatch repair deficient strains CM1330, CM1359 and CM1362

would be slightly less (30% to 45%), however, deletion events would still account for a substantial fraction of the mutations formed. The above calculations are based on the number of in-frame deletions that were scored with our assay giving a Trp⁺ revertant. This may be an under-estimate, as the slower rate of growth of the deletion mutants would mean that their appearance would lag behind that of transversion prototrophs arising at the same time. If this is the case then the deletion pathway could well be the major pathway operating in mismatch repair deficient strains under growth limiting conditions.

It is suggested that the high frequency of deletions we see arise from the conjunction of two circumstances. The first is restricted chromosomal replication associated with starvation for a required amino acid. The *trpA23* conferred auxotrophy is sufficiently leaky that a lawn of residual growth is visible, on minimal agar plates lacking tryptophan, after about seven days. In this case it is correct to say that replication is restricted rather than absent. Jayaraman (1995) looked at post plating mutagenesis in several amino acid auxotrophies in the K-12 strain AB1157. He suggests that leakiness is a requirement for starvation associated mutagenesis and in his system induces leakiness, in the *thr-1* and *leuB6* alleles, using streptomycin to lower translational fidelity. In *argE3* the allele cannot be classified as leaky and shows a much lower rate of post plating mutation with the conclusion that some protein synthesis and concomitant DNA replication is necessary for starvation associated mutagenesis.

The second circumstance is the persistence of mismatched regions of DNA, this would seem to be the logical result of inactive mismatch repair in the *mutY*, *mutH*, *mutL* or *mutS* strains. These regions would either be with single damaged bases i.e. 8-oxo-dG:A, usually repaired by MutY (Michaels *et al.*, 1992a; 1992b, Moriya and Grollman, 1993, reviewed by Tchou and Grollman 1993), or normal non-complementary bases or small loops of DNA usually repaired by the MTHLS system (Kramer *et al.*, 1984; Dohet *et al.*, 1985; Su *et al.*, 1988). While MMR can efficiently repair DNA loops of up to 4 base pairs (Dohet *et al.*, 1986; Learn and Grafstrom, 1989; Parker and Marinus, 1992; Carraway and Marinus, 1993) there was no evidence that it has any activity on larger loops of say six to 27 base pairs (Dohet *et al.*, 1987; Carraway and Marinus, 1993). However, recently Fang *et al.* (1997) have shown that MMR may have a very low activity on larger loops *in vitro* but this repair activity declines very rapidly with increasing loop size. It is possible, therefore, that MMR could display a very weak activity on larger loops under conditions of restricted growth where replication and indeed transcription are infrequent. Such activity may result in excision of one strand of DNA allowing the other strand to adopt secondary structure, synthesis across the secondary structure might then lead to a deletion. Since neither MutY nor the MutH, L or S proteins are necessary for deletion formation, if loops are an essential factor they must be processed by other pathways.

How would the apparent requirement for persisting mismatches and dysfunctional mismatch repair relate to the deletions that were found in the parental strain, IC3126, which is mismatch repair proficient? In starving bacteria, the levels of the MutS and

MutH proteins have been found to decrease (Feng *et al.*, 1996). However, the level of DNA replication also decreases under these conditions, so these proteins may maintain their proper ratio with respect to DNA mismatches produced during DNA replication. Recently Harris *et al.* (1997b) have shown that functional MutL becomes limiting, during stationary phase, for mismatch repair activity despite the apparent maintenance of MutL protein levels. It may be that starving cells are indeed functionally mismatch defective despite apparently having adequate amounts of the three proteins to deal with the level of mismatches produced. Schaaper and Radman (1989) suggested that MutL might be expended during mismatch repair. The protein would remain present in the cell, after use and prior to break down, but be functionally useless to the cell.

Alternatively, MutL may be titrated out by some mechanism thereby reducing its availability to participate in mismatch repair. Titration of mismatch repair proteins has been found in systems where the DNA polymerase error rate was increased to saturate mismatch repair, either through use of a mutator allele (Schaaper, 1988; Damagnez *et al.*, 1989; Schaaper and Radman, 1989; Schaaper *et al.*, 1989) or mutagens (Cupples *et al.*, 1990). Titration was also achieved by the over-production of single stranded DNA with regions of secondary structure containing mismatches (Maas *et al.*, 1994; 1996). In this case, it appeared to be the level of MutS that was affected although since both MutS and MutL are required for functional mismatch repair the result may be the same. Our system already has very slow growth associated with it and concomitant DNA synthesis must be occurring. Another alternative is that this DNA synthesis, occurring under adverse conditions, is

unusually error prone thus saturating the reduced mismatch repair ability as suggested above. If MMR were compromised, mismatches would persist in the DNA despite the cells being genotypically MMR⁺ and would contribute one of our prerequisites for deletion formation.

The strains used contain mutations in several genes involved in damage repair i.e. *umuDC* and *uvrA* all of which are responsive to the SOS regulon. We have also looked at a further gene *recA*, which is central to control of SOS induction, the gene product is involved in cleavage of the LexA repressor protein. It was found that a *recA* deletion did not appear to have any effect on deletion formation in IC3742, although it did effect the number of mutants arising over time on starvation plates. By removing functional RecA, we prevented induction of the SOS response and the induction of all the genes controlled by this regulon. There is recent evidence from Taddei *et al.* (1995) that the SOS response is induced in aged colonies on agar plates and may be important in survival and in the production of mutations in starving cells. While there are at least 26 chromosomal genes regulated by LexA, only two are necessary to promote mutagenesis in *E.coli* namely *umuC* and *umuD* (Sommer *et al.*, 1993). However since the strains already carry a deletion through *umuDC*, any effect of *recA* we see cannot be due to differential mutagenic bypass of lesions in the DNA.

While *recA* strains are viable, mutants grow almost as well as wild type strains, they are very sensitive to forms of damage that block DNA replication and that remain unrepaired. If a damaged chromosome is segregated during division, the resulting daughter will be non-viable. This lethal segregation would explain the observation

that a substantial lawn was still formed on plates bearing the *recA* deletion strain despite the observed decrease in viability. While 8-oxo-dG does not itself block DNA replication (Wood *et al.*, 1990; Shibutani *et al.*, 1991), there may be other forms of lethal oxidative DNA damage, for example thymine glycols or formamidopyrimidines, that will kill cells unable to repair or bypass them during replication. Taking thymine dimers as another example of damage that blocks replication, a *uvrA recA* double mutant can be killed by a single lesion per chromosome compared to the 50 lesions required to kill a *recA*⁺ excision repair defective cell (Friedberg *et al.*, 1995).

When a polymerase encounters a blocking lesion it will stall and replication re-initiates, perhaps several thousand nucleotides downstream (Rupp and Howard-Flanders, 1968; Khidhir *et al.*, 1985). The resulting gap is usually repaired by RecA-dependent homologous recombination, with the sister duplex, during post-replication repair and is usually considered error free. However, recent results would tend to modify this statement. Recombinational repair may not be an inherently error free mechanism but the mismatch repair system serves to monitor the fidelity of the process during strand exchange. In the absence of mismatch repair recombination may occur between sequences that have diverged by perhaps 15-20% (Rayssiguier *et al.*, 1989; Worth *et al.*, 1994). If deletions are occurring in mismatch repair deficient cells at the rate observed and as a generalised process, most of them will also be lethal to the $\Delta recA$ host unless repaired. Thus, recombination may be the way that bacteria maintain their genome integrity when faced with a process that tends towards genomic instability. There is usually more than one copy of the bacterial

chromosome present in individual cells even under stationary phase conditions (Boye and Løbner-Olesen, 1991; Åkerlund *et al.*, 1995). Availability of homologous regions of DNA would give bacteria the opportunity to use homologous recombination to repair chromosomal deletions and restore viability. Since all the small in-frame deletions found allow growth, they may replicate and segregate before they can be repaired even in *recA*⁺ backgrounds.

The other two repair deficiencies in the *trpA23* strains, *uvrA* and *umuDC* can be considered next. There is a low constitutive level of the excision repair genes present in SOS uninduced cells, about 20 molecules of UvrA (Kenyon and Walker, 1981) compared to about 16 molecules of UmuC and 180 molecules of UmuD (Woodgate and Ennis, 1991; Woodgate and Levine, 1996). UvrA increases to about 200 molecules per induced cell (Lin and Sancar, 1992) while UmuC and UmuD are induced to about 200 and 2400 molecules per cell respectively (Woodgate and Ennis, 1991). If, as Taddei *et al* (1995) claim, SOS is induced in starving cells the levels of UvrA, UmuC and UmuD may be intermediate between the basal and induced levels in starving bacteria and may influence the number of mutants we see. UvrA is the damage recognition subunit of the NER system. It has a wide repertoire of damage that it targets for excision, ranging from covalently modified bases to non-covalent drug-nucleotide adducts (Lambert *et al.*, 1989; Snowden *et al.*, 1990; Selby and Sancar, 1991) to apurinic/apyrimidinic sites (Lin and Sancar, 1989).

There is no evidence that 8-oxo-dG is a substrate for the NER pathway (Wagner *et al.*, 1997), although Czczot *et al.* (1991) found that the UvrABC exinuclease was

involved in the repair of lesions induced by methylene blue (MB) and visible light. As 8-oxo-dG was the only damaged base identified in MB-light treated DNA (Floyd *et al.*, 1989; Schneider *et al.*, 1990) and the damage formed was a substrate for the product of the *mutM* gene (8-oxoguanine DNA glycosylase also called formamidopyrimidine DNA glycosylase), it was suggested that UvrABC was acting upon this damage. However, in light of other results (Boiteux *et al.*, 1992; Ravanat and Cadet, 1995) it is likely that the genotoxic lesion is something other than 8-oxo-dG and it is this lesion that is a substrate for NER.

Deletions were found in both the *uvrA*⁺ and *uvrA*⁻ backgrounds, when combined with *mutY*, so the gene product was obviously not essential in their formation. However, since UvrA does recognise other forms of damage e.g. thymine glycols (Lin and Sancar, 1989; Kow *et al.*, 1990), it may have an effect on mutagenesis in starving bacteria if they are prone to accumulate excess oxidative damage. In our *trpA23* system, *uvrA* alone had no noticeable effect on the number of mutants that arose on starvation plates. This agrees with the results of Bridges (1993) who looked at reversion of an ochre mutation in *trpE* but disagrees with the results of Hall (1995). Hall found that elements of NER were "selection-induced-specific mutator genes" increasing the post plating mutation rate of a *trpA46* allele by a factor of 80. Hall suggested that in starving cells there is accumulation of damage that is susceptible to repair by NER and is mutagenic. The *trpA46* allele normally reverts at an A:T site (Yanofsky *et al.*, 1966b), if this is also the case under starvation conditions it may be a damaged form of adenine or thymine, rather than guanine, that is responsible for the enhancement seen by Hall. There is difficulty in this interpretation. As the ochre

strains in Bridges system should also respond to damaged adenine or thymine but no effect is seen, one has to conclude that the effect, if real, is locus specific.

There is evidence from plasmid experiments (data not shown), combining UmuDC in a NER proficient and deficient background, that UvrA may be responsible for removing some forms of damage, under starvation conditions, that are otherwise either lethal or mutagenic. UmuDC is involved, along with RecA, in the error prone translesion bypass of DNA damage that blocks replication (Bridges and Woodgate, 1984; 1985a; 1985b). Strains proficient in error prone repair were noticeably more mutagenic than their *umuDC*⁻ parents. This result implies that there are forms of damage, produced in starving cells, which can be recognised by UvrA and are removed by the NER system. However, when NER activity is absent, this same damage can be bypassed by UmuDC resulting in increased mutagenesis. Overexpressing UmuDC might be expected to increase mutagenesis, perhaps because there are other processes occurring in starving cells that will out compete UmuDC to repair damage, possibly RecA dependent post-replication recombination repair.

It is not in the cells interest to promote error prone repair and there are a number of control mechanisms to ensure that this pathway is the method of last resort. UmuD must be activated to UmuD' to participate in error prone repair, the RecA protein (Nohmi *et al.*, 1988) accomplishes this. We can speculate that if a plasmid constitutively expressing *umuD'* was introduced into a *uvr*⁻ background then mutagenesis would be further increased. For there to be a noticeable effect in our

system there must be activation of the UmuD protein, perhaps supporting the claim of Taddei *et al.* (1995) that there is SOS induction in starving cells. An important point to note here is that in the *trpA23* system as well as that of Taddei *et al.* (1995) protein synthesis can occur, albeit at a low level, as both systems are leaky. Protein synthesis is a vital component of SOS induction and it is possible that SOS can only be of importance in nutritionally stressed bacteria where some protein synthesis can occur *de novo*.

A possible role for SOS also agrees with the observations of Bridges (1993) who found that in a *uvr⁻* SOS constitutive strain expressing activated RecA*, the number of slow growing Trp⁺ mutants arising over time was increased. The SOS system has also been found to play a part in deletion formation in a plasmid system used by Balbinder *et al.* (1993). They found that overproduction of activated RecA* and UmuC, in a SOS constitutive background, increased the spontaneous deletion frequency of palindromic inserts in a plasmid gene. Overproduction of RecA was not found to affect the deletion rate of non-palindromic inserts perhaps indicating the existence of two separate mechanisms. Their system, as with most experimental systems, examined deletions in growing cells or in cells that had only spent a brief time in stationary phase. The *trpA23* system in contrast looked at deletion formation in cells that had spent a prolonged period under conditions of nutrient privation. It is possible that in starving cells pathways may display activities that are not detectable in short term assays so RecA and UmuDC may affect frequencies of palindromic and non-palindromic deletion formation although we found no effect of $\Delta recA$ or $\Delta umuDC$ on deletion frequency.

Many (75%) of the deletions recovered in *trpA* were flanked by direct repeats varying from a single base pair up to four bases. Terminal direct repeats are known to play a role in deletion formation (Farabaugh *et al.*, 1978; Albertini *et al.*, 1982a; 1982b; Mazin *et al.*, 1991; Chédin *et al.*, 1994) perhaps stabilising secondary structures or providing regions of homology during slippage and mispairing of replicating DNA. Contact between direct repeats could facilitate recombination but it is known to be RecA independent (Jones *et al.*, 1982; Collins *et al.*, 1982; DasGupta *et al.*, 1987; Mazin *et al.*, 1991; Lovett *et al.*, 1994), perhaps because the repeats are below the minimum length (30-70 base pairs) required for RecA mediated homologous recombination (Watt *et al.*, 1985; Shen and Huang, 1986; King and Richardson, 1986). This is consistent with the observation that RecA had no effect on deletion formation in *trpA* but did affect viability and mutagenesis under our conditions.

Factors that affect the frequency of deletion between repeats include: repeat length, GC content or thermal stability and proximity of repeats to each other (DasGupta *et al.*, 1987; Williams and Müller, 1987; Peeters *et al.*, 1988; Weston-Hafer and Berg, 1989; 1991; Pierce *et al.*, 1991; Sinden *et al.*, 1991; Trinh and Sinden, 1993; Chédin *et al.*, 1994). The repeats flanking the *trpA23* deletions vary from a single base pair up to four base pairs and are thus shorter than the repeats observed in most other systems (four to nine base pairs). It is interesting to note that of the 12 different deletions with terminal repeats, 10 were predominately composed of GC base pairs. The deletions were all in a defined region of *trpA* and the largest deletion observed spanned 27 base pairs so the terminal repeats in these cases were in close proximity.

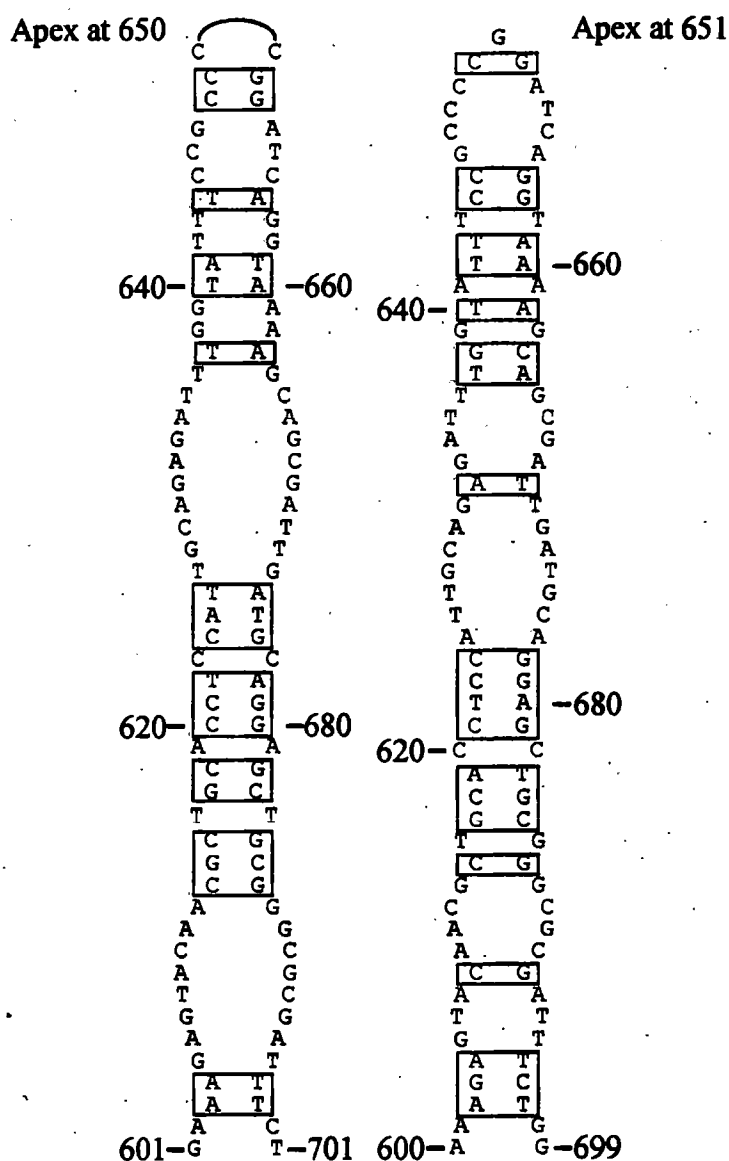
By no means all our deletions were flanked by repeats, 25% had no homology at either terminus. Perhaps in these cases more complex secondary structure was involved in their formation. There proved to be two potential pseudo-hairpins in the region of the *trpA23* mutation Figure 3.13.1. These structures are not perfect hairpins and the formation of real secondary structure in actively growing cells must be suspect. However, in starved cells where the frequencies of DNA replication and transcription both processes that have the potential to "iron out" secondary structure are much reduced we cannot rule out *in-vivo* formation of these or similar structures. A proposed model that secondary structure can explain deletions, by juxtaposition of deletion endpoints, would account for the apparent absence of repeat termini in some of our deletion sequences (Ripley and Glickman, 1983; Glickman and Ripley, 1984). In this model, ligation and replication across the base of the secondary structure, with subsequent rounds of replication creating homologous DNA strands bearing the deletion was suggested.

Alternatively, the secondary structure may be processed to produce deletions. It has been known for some time that plasmids or phages carrying inserts containing large palindromic structures are unstable in *E.coli* (Collins *et al.*, 1982; Hagan and Warren, 1983).

Leach and Stahl (1983) found that strains deficient in recombination nucleases were able to maintain palindromic structures and it was subsequently discovered that the only genes required to be dysfunctional were *sbcDC* (Chalker *et al.*, 1988; Gibson *et al.*, 1992; Leach, 1996).

Figure 3.13.1

Putative hairpin structures in the region of the *trpA23* mutation



Two putative pseudohairpin structures have been found in the region of the *trpA23* mutation. Only one strand of DNA is shown for each structure. Numbers refer to the *trpA* coding sequence, position 1 taken as A in the ATG initiation codon. The stability or formation of such secondary structure *in vivo* is uncertain, however, should they form even transiently they may provide targets for mismatch repair systems or for nucleases involved in maintenance and stability of secondary structure e.g. the SbcDC nuclease.

SbcDC has recently been shown to comprise a processive double strand exonuclease (Connelly *et al.*, 1997) that targets and resolves cruciform structures in DNA. Because of the putative secondary structure in the region where our deletions occur, we looked at the effect of $\Delta sbcDC$ on deletion formation. Although there was no effect of this endonuclease on deletion formation the effect of other recombination endonucleases such as RecBCD or SbcB cannot be discounted. If the secondary structure is important perhaps it is processed by some other means or is important in a purely structural way *i.e.* the secondary structure itself is sufficient to target deletion formation to this region of DNA.

How do we reconcile the two mismatch repair deficiencies, MutY and MutHLS, producing the same endpoint? Do they operate by one or more different mechanisms? Results in other systems suggest that there are at least two major pathways for deletion formation. One is the "copy-choice" pathway originally proposed by Streisinger *et al.* (1966), where strand slippage occurs during DNA replication and usually involves deletions formation between short repeats (Albertini *et al.*, 1982a; 1982b; Dianov *et al.*, 1991; Mazin *et al.*, 1991; d'Alençon *et al.*, 1994; Bi and Liu, 1994; Lovett and Feschenko, 1996). The other process involves enzymes that cut and join DNA such as topoisomerases and do not involve homologies (Shimizu *et al.*, 1997; Uematsu *et al.*, 1997). Since both types of deletion event were found in our system, both models need to be considered.

The viability results with the *recA* deletion strain suggest that there is damage, occurring in starving cells, that blocks replication and is fatal if not repaired. Replication blocking damage, for example AP-sites, would lead to pausing or even collapse of replication forks and there is evidence that even if the replication fork

survives the replisome complex is slowed to some degree. This type of event has been heavily implicated in deletion formation (Asai *et al.*, 1994; Bierne and Michel, 1994; Kuzminov, 1995a; 1995b; Bierne *et al.*, 1997a).

In this context, it would be interesting to look at the effect of the RecF protein in our system. This protein has previously been identified as a component in recombination repair and more recently has been shown to be important to the reconstitution of the replisome after an encounter with a blocking lesion (Courcelle *et al.*, 1997). However unlike the deletions recovered in the *trpA* gene, Bierne *et al.* (1997b) have shown that the activation of a RecF dependent pathway for enhanced deletion formation between tandem repeats is also dependent on RecA. It is probable that *recF* mutants would have lowered viability during starvation experiments, but if enough mutants were examined it should be possible to tell whether this protein is important to deletion formation and whether replication fork collapse is important in our system. Saveson and Lovett (1997) have found that an *E. coli* strain mutant in the ϵ editing sub-unit of Pol III (*dnaQ*) caused a large increase in deletion formation between large tandem repeats. Mutants in *dnaQ* are associated with strong mutator phenotypes (Echols *et al.*, 1983; Schaaper and Radman, 1989; Krishnaswamy *et al.*, 1993) due to a large increase in the number of base substitution mutations. Absence of the editing function would lead to persistence of mismatches in the newly synthesised DNA and presumably more importantly, to us, to a reduction in the fidelity of template choice. Other mutants in Pol III also increased deletion formation of large repeats possibly by slowing the replication rate, a process which may favour strand slippage (Bierne *et al.*, 1997c; Saveson and Lovett, 1997).

These two factors are reminiscent of the situation found in our starving mismatch repair deficient cells. Replication would be expected to proceed at sub-optimal rates in nutritionally deprived bacteria perhaps because the supply of dNTP's, energy or a required protein is limiting. The initiating event in deletion formation would thus be solely due to pausing of the advancing replication fork allowing transient separation of the newly synthesised strand from the template. Re-annealing of the separated strand would soon take place and could result in a deletion if the re-annealing is erroneous. The ϵ sub-unit of Pol III recognises and edits mismatches, it would probably have the effect of terminating illegitimate recombination events, through inappropriate choice of template. Obviously, its absence would increase the likelihood of illegitimate recombinants persisting and leading to deletions. The ϵ sub-unit does not recognise all replicative errors, some are repaired by MMR and MutY, as demonstrated by the difference in observed error rates in wild type and mismatch defective cells.

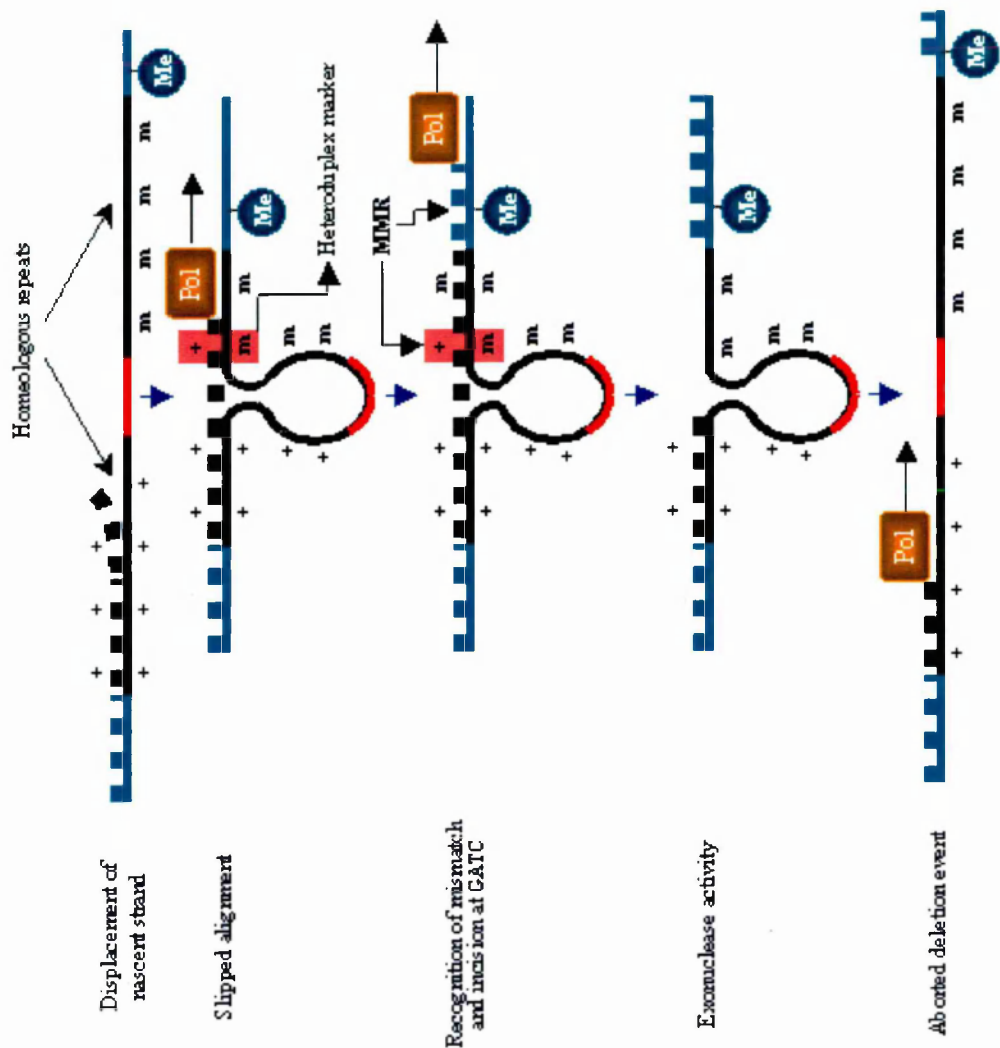
Prevention of illegitimate copy-choice templates would also be expected to have a component of mismatch correction associated with it. Functional mismatch correction may have the effect of terminating such events, recognising and excising the mismatched region, subsequent re-synthesis would more than likely abolish the deletogenic event Figure 3.13.2. Again it would be interesting to observe the effect of a *dnaQ* and MMR combined deficiency on deletion formation. Since a cumulative effect of a *mutS* and a *dnaQ* mutation has been observed in a system looking at simple repeat sequence stability (Strauss *et al.*, 1997), one would predict that the deletion rate in the *trpA23* system would also be increased above that shown by

Prevention of deletogenesis by MMR

A putative mechanism whereby MMR can abort deletogenic events between homeologous repeat sequences. During replication or repair synthesis, the 3' end of the nascent strand becomes detached from the template strand. There is now the possibility that it can misalign with short lengths of homology, possibly mediated by recombination proteins such as RecA, in heterologous sequences. Continuation of synthesis leads to a deletion event. Since the new strand is hemi-methylated, it is a substrate for MMR mediated repair, which can act on, mismatches generated by pairing of the heterologous sequences. Incision at the nearest GATC site followed by excision of the intervening region may destabilise and abort the deletion process allowing correct resynthesis of the DNA. A similar result would occur if the 3' exonuclease proof-reading subunit of DNA polymerase III accomplished the mismatch recognition and strand degradation steps. Adapted from Lovett and Feshenko, 1996.

Figure 3.13.2

Prevention of deletogenesis by MMR



either single deficiency. A caveat to this prediction is that growth media appears to modulate the activity of certain *dnaQ* mutations (Krishnaswamy, 1993), with mutagenesis levels on minimal media being very much lower than those observed on rich media. In the *trpA23* system, some if not most of the mutagenic events undoubtedly occur on minimal agar plates, in this case would there be any observed increase in the deletion levels seen?

It is possible that illegitimate recombination events are relatively common in replicating cells, especially if the replicative rate is slower than optimal. Functional *dnaQ* DNA polymerase error correction mechanism and mismatch repair may be vital to prevent such illegitimate recombination occurring as well as to prevent base pair substitution mutations. One can see that this mechanism would account for the situation where short repeats have been found, these provide the homology for the copy-choice process to work, however our deletions do not all exhibit such repeats. Are these remaining deletions the result of a different mechanism?

Bierne *et al.* (1997a) have recently reported an effect of DNA topoisomerase I on deletion formation where no direct terminal repeats were found, in contrast a *topA* mutant had no effect on deletion rate between direct repeats with 3-10 base pair homology. In this system replication pausing was also considered to be important, as deletion termini were associated with sequences that terminated replication in *E.coli*. Topoisomerase I is interesting as it displays a structural preference for cleavage sites, near to the junction of single and double stranded DNA (Kirkegaard and Wang, 1984). One can speculate that as well as stalled replication forks, secondary structure

would also provide regions of single and double stranded DNA but whether the enzyme would recognise such structures is unknown. However, since Topoisomerase I displays both cleavage and ligation activities, it does have the means to cut and rejoin DNA molecules both functions would be necessary to form viable deletion mutations. *E.coli* DNA gyrase has also been demonstrated to increase short homology independent recombination (Shimizu *et al.*, 1997).

It is harder to associate deficient mismatch repair with deletions that apparently do not require homology and are not therefore susceptible to homeologous recombination. We are left with the observations that there is apparently no requirement for terminal homology yet mismatch deficiencies stimulate deletion formation. While mismatch repair deficiencies significantly enhance RecA dependent homeologous recombination (Rayssiguier *et al.*, 1989; Petit *et al.*, 1991; Worth *et al.*, 1994; Zhart *et al.*, 1994; Humbert *et al.*, 1995), in the *trpA23* system the process was shown to be *recA* independent. Mismatch deficiencies undoubtedly enhance deletion formation in the *trpA* gene it is not possible to provide a complete explanation as to the way in which they do so.

The final point to consider is why are deletions observed in this particular region of the *trpA* gene. The system studied depends on the formation of Trp⁺ revertants that form colonies and that can be isolated for sequencing. All the deletions seen are in-frame events, if the deletions were irrelevant to the Trp⁺ phenotype we would have expected to find out-of-frame events as well as larger deletions than the ones observed. This argues that it is the deletions that are responsible for the observed

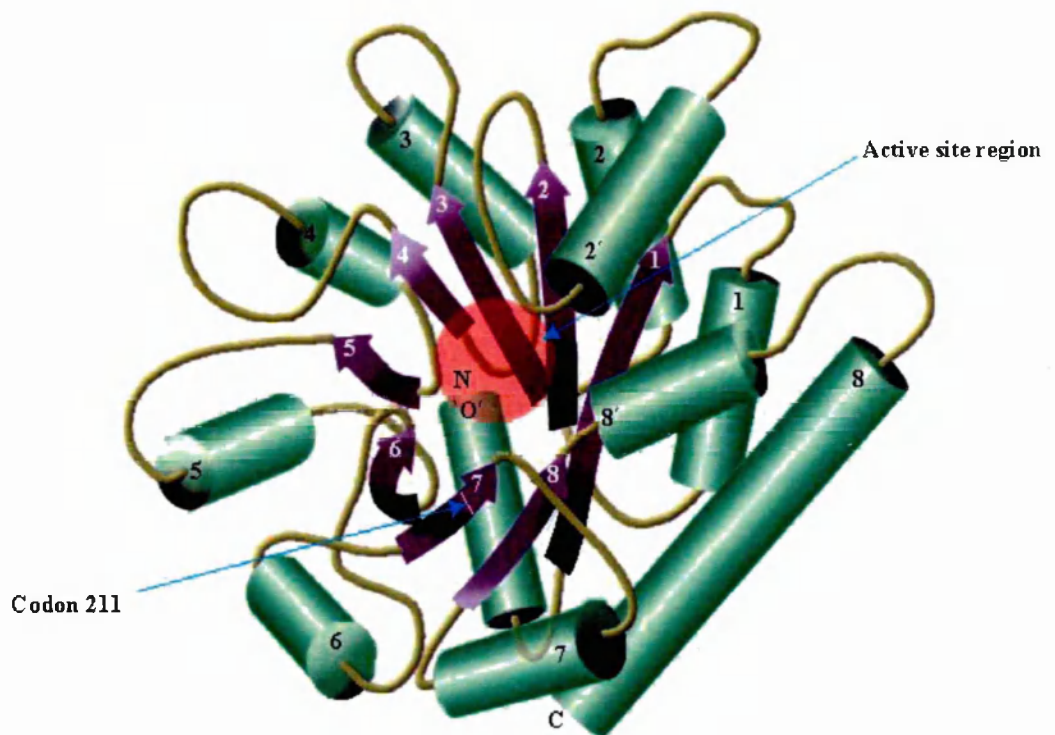
phenotype. The three dimensional structure of the *Salmonella typhimurium* tryptophan synthase α sub-unit has been determined (Hyde *et al.*, 1988). A comparison of the *E.coli* and *S.typhimurium* TrpA amino acid sequence indicates that they are similar in the region of interest *i.e.* residue 211 which forms part of the β -sheet 7 and α -helix7 (Nichols and Yanofsky, 1979). The *trpA23* mutation results in a change from glycine at codon 211 to arginine. Murgola (1985) lists nine amino acid substitutions at this particular site that will give a functional reversion. They vary considerably in size but all except one lack a polar terminal group the exception, asparagine, is relatively small.

There are eight other amino acids that are known to result in a dysfunctional protein, all of these possess a polar side group and/or a bulky ring moiety. It is suggested that these residues confer inactivity due to steric hindrance of the active site or ionically interfere with the access of indole 3-glycerol-phosphate to the active site.

Thus the function of the small in-frame deletions maybe to restore activity by lessening the proximity of the charged or bulky residues to the active site. The structure of TrpA is shown in Figure 3.13.3, the *trpA23* mutation is situated on β -sheet-7, all except one of the deletions occur in α -helix-7 or the adjacent part of β -sheet-7. The effect of these deletions may be to pull the β -sheet and the interfering residue away from the active site. The exceptional deletion results in the removal of the *trpA23* site and thus removes the arginine residue from the protein. It is known that as little as 2% of wild-type tryptophan synthase α activity confers virtually wild-type growth rate (see Murgola, 1985).

Figure 3.13.3

Structure of the α subunit of Tryptophan synthase from *Salmonella*



The structure of the TrpA protein from *Salmonella* was elucidated by Hyde *et al.* (1988), β strands are shown as purple arrows and α helices are green cylinders. Numbers refer to the positions of β sheets and α helices with 0 being the amino terminus of the protein up to α helix 8 at the carboxyl terminus. The diagram shows the position of codon 211 (site of the *trpA23* mutation) in β strand 7. Deletions occur in the region encompassing codon 211 extending to the proximal portion of α helix 7. The active site is indicated by the red circle. Deletions are assumed to decrease the interference of amino acids at codon 211 (either from charge or bulky side groups) in the access to or functionality of the active region.

Since the deletion strains have a slow growing phenotype the activity of the mutant tryptophan synthase α protein must be relatively inefficient. The occurrence of the deletions to this particular region of the protein would seem to be related to their function in suppressing the effect of the arginine residue at codon 211, although the efficiency with which they restore function to the protein is low.

3.2 *tas*, a gene suppressing the tyrosine auxotrophy of the *Escherichia coli* B strain WU3610

When about 3×10^8 cells of the *E.coli tyrA14* strain WU3610 are plated to minimal agar plates lacking tyrosine, slow growing tyrosine independent colonies begin to arise from day six onwards and eventually number several hundred colonies per plate. Reversion at the *tyrA14* allele and at known suppressor loci has previously been ruled out (Bridges, 1994, and Appendix I). Therefore, attempts to directly isolate the unknown suppressor by virtue of its complementation of tyrosine auxotrophy were made. A genomic library was constructed from two tyrosine independent colonies and the gene complementing the *tyrA14* auxotrophy was isolated and sequenced. The gene subsequently called *tas* was shown to be absolutely required for the process of starvation-associated mutation to tyrosine prototrophy in WU3610. However, *tas* does not appear to be the mutational target responsible for the formation of the slow growing colonies as no sequence change or amplification of the *tas* gene was found. The process may therefore require a mutation in another gene possibly involved in regulation of *tas* expression.

3.21 Methods

Bacterial strains and plasmids

Bacterial strains and plasmids are shown in Tables 3.21.1 and 3.21.2. Strains WU3610_(old) and WU3610_(new) are specifically considered in Appendix II. P1(cml) transductions were carried out as previously described with selection for the appropriate antibiotics. Transduction of the *pheA13::Tn10* allele was verified by the inability of strains to grow on minimal media lacking phenylalanine. Transduction of the Δ *tas::Km^R* allele was verified using PCR, with primers SAMPCR1 (5'ACAAATAAGGTCAGCATCCGGCTGGCC3') and SAMPCR2 (see below), the deletion allele produced a fragment approximately 400 base pairs larger than the wild type allele. Transduction of *rpoS::Tn10* was verified using the H₂O₂ bubbling assay. Bacterial culture conditions were as previously described. Starvation-associated mutation to tyrosine independence was observed when around 3×10^8 bacteria were plated to minimal agar plates containing glucose and leucine. Whole plate wash off was used in viability determinations, where 1×10^6 or 3×10^8 bacteria were plated on minimal agar lacking tyrosine or leucine and viability followed for four days. Antibiotic concentrations were as previously described Table 2.1.

Construction of tyrosine complementation libraries

Two slow growing tyrosine independent mutants (SAM4 and SAM6) were isolated from day eight of a starvation-associated mutation experiment. Single colonies from

Table 3.21.1

Bacterial strains

Strain	Relevant genotype	Source or derivation
AB1157	<i>argE3</i> _(oc) , <i>hisG4</i> _(oc)	T.Kato
B _(Alper)		Tikvah Alper
B _(CSH)		John Donch
DH5αF'	<i>recA1, endA1, gyrA96, hsdR17</i> (rk ⁻ , mk ⁺)	Laboratory stocks
KA12	Δ(<i>pheA-tyrA-aroF</i>)	Kast <i>et al.</i> , (1996)
MT1	<i>rpoS</i> ::Tn10	F.Taddei
N3078	<i>pheA13</i> ::Tn10	R.Lloyd
WP2	<i>trpE65</i> _(oc)	E.M. Witkin
WU3610 _(old) ^a	<i>tyrA14</i> _(oc) , <i>leu308</i> _(am) , <i>rpoS</i> ?	E.M. Witkin
WU3610 _(new) ^a	<i>tyrA14</i> _(oc) , <i>leu308</i> _(am)	R.Bockrath
WU3610 <i>mutL</i> ::Tn10	as WU3610 _(new) but <i>mutL</i> ::Tn10	R.Bockrath
CM1338	as WU3610 _(old) but <i>pheA13</i> ::Tn10	this work P1 (N3078) × WU3610 _(old)
CM1355	as WU3610 _(old) but Δ <i>tas1</i> ::K ^R <i>m</i>	this work see materials and methods

Table 3.21.1

Bacterial strains

Strain	Relevant genotype	Source or derivation
CM1376	as WU3610 _(new) but <i>rpoS</i> ::Tn10	P1 (MT1) × WU3610 _(new)
CM1415	as WU3610 _(new) but <i>ΔtasI</i> :: <i>Km</i> ^R	this work P1 (CM1355) × WU3610 _(new)
CM1421	as WU3610 _(old) but <i>mutL</i> ::Tn10	this work P1 (WU3610 <i>mutL</i> ::Tn10) × WU3610
CM1422	as CM1355 but <i>mutL</i> ::Tn10	this work P1 (WU3610 <i>mutL</i> ::Tn10) × CM1355
CM1428	as CM1415 but <i>rpoS</i> ::Tn10	this work P1 (MT1) × CM1415

^a see Appendix II.

Table 3.21.2

Plasmids

Plasmid	Markers or construction ^a	Source
pACYC184	Cm ^R , p15A replicon	New England Biolabs
pGEM-3Zf(-)	Ap ^R , <i>colEI</i> replicon	Promega UK
pKCMT-W	Ap ^R , <i>aroH</i> , <i>colEI</i> replicon	Kast <i>et al.</i> , (1996)
pKIMP-UAUC	Cm ^R , <i>pheC</i> , <i>tyrA</i> , p15A replicon	Kast <i>et al.</i> , (1996)
pMAK705	Cm ^R , pSC101 (ts) replicon	Hamilton <i>et al.</i> , (1989)
pUK4K	Ap ^R , Km ^R GenBlock	GibcoBRL
pART34	as pGEM-3Zf(-), <i>tas</i> orientation I	this work
pART35	as pGEM-3Zf(-), <i>tas</i> orientation II	this work
pART36	as pART34 (<i>KpnI</i> - <i>PstI</i>)	this work
pART37	as pART34 (<i>SspI</i> - <i>PstI</i>)	this work
pART38	as pART34 (<i>EcoRV</i> - <i>PstI</i>)	this work
pART39	as pART34 (<i>PstI</i> - <i>EcoRV</i>)	this work
pART40	as pART34 (<i>SspI</i> - <i>Bst</i> 11071)	this work
pART42	as pART34 (<i>SspI</i> - <i>HpaI</i>)	this work

Table 3.21.2

Plasmids

Plasmid	Markers or construction ^a	Source
pART47	as pART36, $\Delta EcoRV$ - <i>Bst</i> I 1071 insertion <i>Bam</i> HI linker	this work
pART48	as pART47, insertion of <i>Bam</i> HI- <i>Bam</i> HI GenBlock	this work
pART50	as pMAK705, insertion of $\Delta tas1::Km^R$	this work
pART51	as pACYC184, insertion of <i>tas</i>	this work
pART52	as pKIMP-UAUC, $\Delta pyrA$ insertion of <i>tas</i>	this work

^a Precise derivation of plasmids is given in the methods section.

each were grown in nutrient broth with shaking. Chromosomal DNA was extracted and digested with *Pst*I. The resulting fragments were ligated, in a two to one ratio, with alkaline phosphatase treated *Pst*I digested pGEM-3Zf(-). The ligation mixture was transformed, into electrocompetent WU3610, according to the protocol of Dower *et al.* (1988). After expression (one hour) the electroporation mixtures were spun and washed once with phage buffer and resuspended in 1ml of phage buffer. The entire transformation was plated to minimal agar maxi-plates containing ampicillin and leucine. The plates were incubated at 27°C for 48 hours. Plasmid DNA was isolated from several *Tyr*⁺ colonies from each transformation and transferred to DH5 α F'. Two plasmids bearing the insert in opposite orientations were selected, pART34 and pART35, and single stranded DNA isolated. The plasmid inserts were sequenced in their entirety; primers were synthesised according to sequence information obtained by primer walking.

Construction of deletion plasmids

Using restriction endonuclease sites identified during sequencing, deletion plasmids were constructed. All the insert fragments were derived from pART34 and were subcloned into the MCS of the pGEM-3Zf(-) vector. Plasmid pART36 was constructed by inserting the 1.84 kb *Kpn*I-*Pst*I fragment into the identical sites in the vector. Similarly, the 1.37 kb *Ssp*I-*Pst*I fragment was cloned into *Pst*I-*Eco*ICR sites to form pART37, the 1.14 kb *Eco*RV-*Pst*I fragment into *Pst*I-*Eco*ICR sites to form pART38, the 1.09kb *Pst*I-*Eco*RV fragment into *Pst*I-*Eco*ICR sites to produce pART39, the 1.06 kb *Ssp*I-*Bst*11071 fragment into the *Eco*ICR site to produce

pART40 and the 1.18 kb *SspI-HpaI* fragment into the *EcoI*CR site to produce pART42.

PCR of the chromosomal *tas* gene

Boiling steps were carried out as previously described. PCR conditions were as previously described and PCR was run for 30 cycles. Reactions contained 100pmole of primers SAMPCR2 (5'CGCTGTGGATCTGGCAGCGCCGTCAT3') and SAMPCR3 (5'XCCGGGTGTTCCAGTGATTACGTCATG3'). SAMPCR3 was biotinylated (X) at the 5' end to allow Dynabead separation and sequencing as previously described.

Genetic complementation of tyrosine auxotrophy

The two-plasmid system of Kast *et al.* (1996) consisted of pKCMT-W (mono-functional chorismate mutase activity, *aroH*) and pKIMP-UAUC (mono-functional prephenate dehydrogenase *tyrA* and prephenate dehydratase activities, *pheC*). The *tas* gene was subcloned, from pART34, on a 2 kb *SphI-BamHI* fragment into identical sites in the low copy number vector pACYC184 to produce pART51. The plasmid pART52 was derived from pKIMP-UAUC by excision of the *Clal* fragment, containing the mono-functional prephenate dehydrogenase gene (*tyrA*) and replacement by the *tas* gene on a 2 kb fragment. This fragment, generated by digestion of pART34 with *SphI*, had previously had phosphorylated *SphI-Clal* linkers (5'CATCGATGCATG3') added to both ends. Plasmids were introduced

sequentially into either electrocompetent WU3610 or KA12 by electroporation according to the protocol of Dower *et al.* (1988).

Generating a chromosomal deletion of *tas*

Plasmid pART47 was generated by digestion of pART36 with *Bst*11071 and *Eco*RV, (both endonucleases produce blunt end cleavage products) deleting 822 base pair from the *tas* gene. A unique *Bam*HI site was introduced by insertion of a phosphorylated linker (5'CGCGGATCCGCG3') into the resulting, alkaline phosphatase treated, pART36 fragment. The *Bam*HI fragment from pUC4K, containing the kanamycin GenBlock, was inserted into pART47 to produce pART48. The *tas* deletion construct was subcloned into pMAK705 on a *Kpn*I-*Sph*I fragment to produce pART50. The deletion allele $\Delta tas1::Km^R$ was transferred into the WU3610 chromosome using the protocol of Hamilton *et al.* (1989).

Probing for *tas* gene amplification

Chromosomal DNA was isolated from slow growing Tyr^+ mutants using the Genomic DNA purification kit (Promega (UK) Ltd, Southampton, UK) according to the manufacturers instructions. Five hundred nanograms of DNA were digested with *Pst*II and run on a 1% agarose gel. The gel was blotted according to the protocol of Southern (1975). The filter was probed either with a 1200 base pair $\gamma^{32}P$ end labelled PCR fragment, generated using primers SAMPCR1 and SAMPCR2 for *tas*, or with a 880 base pair PCR product generated using the primers;

RB1(5'CATACCAGTACCAACCAGCG3') and RB6(5'GCGAAGAAATCGAAGGTTCCG3') for a control gene (*rpoB*). Both the *tas* and *rpoB* chromosomal *Pst*I fragments were selected to be approximately 2 kb in size to alleviate any discrepancy in blotting efficiency for different sized molecules.

3.22 Results

In an attempt to isolate the gene responsible for the slow growing tyrosine independent colonies that arose during starvation experiments, Tyr⁺ mutant colonies of WU3610 were isolated from eight-day-old plates. Genomic libraries were constructed from two such Tyr⁺ slow growing mutants (SAM4 and SAM6). The enzyme *Pst*I was used to digest the extracted chromosomal DNA and the resulting fragments were cloned into the high copy number vector pGEM3-Zf(-).

*Pst*I has a six base pair recognition site and on average will cut every 4096 base pairs. The *E.coli* chromosome is about 4.7Mb and would equate to about 1,150 *Pst*I fragments assuming random distribution of the recognition site. If all the cloned fragments transform with equal efficiency, a minimum of 1.15×10^3 transformants (M) would be needed to cover the entire chromosome. In practice it is recommended that at least 10 times this number are plated to ensure a 99.9% probability that all single copy genes are represented in a library. Transformation data for the two libraries are shown in Table 3.22.1. Transformation with the vector, pGEM3-Zf(-), yielded just under 10^9 transformants with a single tyrosine independent colony on the selective plates. This frequency of tyrosine independence, 1.25×10^{-9} , is well within

the range of spontaneous mutation frequencies expected from WU3610. Thus, any effect of either the vector or the competent cells on the number of tyrosine independent colonies arising in either of the other two transformations can be discounted.

Table 3.22.1

Transformation efficiencies of genomic libraries from two slow growing tyrosine independent mutants of WU3610

Library	Total transformants	Trp ⁺ transformants	Frequency ^a Trp ⁺	T/R ^b
control	8.0×10^8	1	1.25×10^{-9}	NA
SAM4	5.05×10^5	463	9.12×10^{-4}	1.05
SAM6	1.10×10^6	779	7.08×10^{-4}	0.81

^a frequency of Trp⁺ colonies compared to total transformants.

^b see results section.

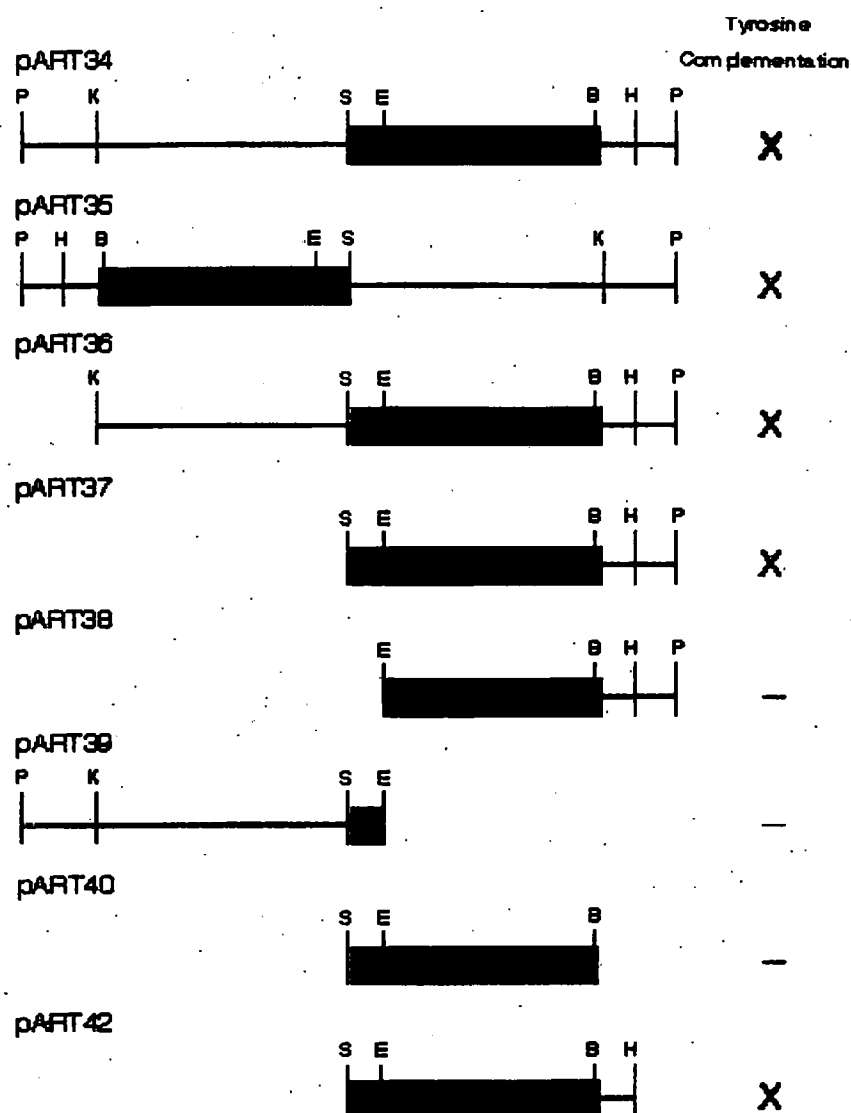
Taking SAM4 as an example, a total of 5.05×10^5 transformants were plated. This is 439 (R) times the minimum number of clones required to theoretically cover the entire chromosome ($5.05 \times 10^5/M$). If complementation is due to a single gene then the expected ratio of the number of tyrosine independent clones (T), on the selective plates, divided by R should be close to 1. For SAM4 the number of tyrosine independent colonies was 463 so T/R equals 1.05, the equivalent value for SAM6 is 0.81. Both values are entirely consistent with the complementation of tyrosine auxotrophy in WU3610 being due to a single gene (fragment).

Plasmids were isolated from several tyrosine independent colonies from both SAM4 and SAM6 transformations. All the plasmids isolated were found to contain identical 2.2 kb *Pst*I inserts and two plasmids, pART34 and pART35 bearing the insert in opposite orientations, were sequenced. Analysis of the sequence revealed that there were two potential open reading frames (ORF) associated with this fragment of DNA. ORF I was 346 residues in length and contained regions 5' to the putative start codon; ORF II was 284 residues long but began only three base pairs in from one end of the insert and so lacked native promoter sequences.

Disregarding read-through from plasmid sequences, it is unlikely that ORF II would form a functional gene in this context and attention was focused on the other coding region. A number of deletion plasmids were constructed, using defined restriction endonuclease sites, and their ability to complement the tyrosine auxotrophy in WU3610_(old) was assessed, the results are shown in Figure 3.22.1. Only plasmids carrying the entire coding region as well as approximately 200 base pairs 5' to the putative start codon were able to complement the auxotrophy. Thus, ORF I had to be responsible for the observed complementation and carry its own promoter region in the DNA immediately 5' to the coding sequence. Additionally, taking A in the *tas* initiation codon (ATG) as position 1, there is a potential Shine-Dalgarno sequence (5'AAGGA3') between positions -8 to -12. It was assumed that the promoter had to be located between the translational start codon and the *Hpa*II site in pART42, this being the smallest construct able to complement tyrosine auxotrophy.

Figure 3.22.1

Deletion plasmids complementing tyrosine auxotrophy in WU3610



Construction of plasmids used to identify the ORF suppressing the tyrosine requirement in WU3610. Cloned genomic DNA is indicated by a thick line. The *tas* ORF is indicated by a shaded box with an arrow showing the direction of transcription. Relevant restriction sites are shown: (B) *Bst*11071, (E) *Eco*RV, (H) *Hpa*I, (K) *Kpn*I, (P) *Pst*I, and (S) *Ssp*I. The ability of each plasmid to complement the tyrosine auxotrophy in WU3610 is shown thus: (X) indicates suppression, (-) indicates no suppression.

Examination of this region indicated that there were potential promoter sequences available. However, they did not closely match either the -10 or -35 consensus sequences but they are the nearest matches that can be identified in this region.

	-35	spacing base pairs	-10
consensus	TTGACA	17	TATAAT
ORF I	AGGTCA	15	TAAGAT

The spacing between the two elements is only 15 base pairs rather than optimal 16-17 base pairs found in most promoters. Utilisation of these two sites as the promoter for this gene has not been verified experimentally. Transcription may well be initiated at a different site, perhaps one with less homology to known promoter sequences or at a site outside the region examined. ORF I has provisionally been given the name *tas* (tyrosine auxotrophy suppressor).

The slow growing Tyr⁺ colonies that arise during starvation experiments take seven to eight days to reach a diameter of approximately 1mm. It was obvious during cloning of *tas* that the plasmids carrying this gene conferred a far faster growth rate on WU3610_(old). Colonies of 3-4 mm diameter grew in 48 hours on minimal agar plates lacking tyrosine. Thus, having identified a gene responsible for complementation of the *tyrA14* auxotrophy when carried on a plasmid, the sequence of the chromosomal gene from wild type WU3610_(old) was determined. Taking the A in the *tas* initiation codon (ATG) as position 1, the sequence from -240 to +1079 in the plasmid and chromosomal genes was found to be identical. Therefore, the

tyrosine independent phenotype observed in strains carrying *tas* on a plasmid was due to the presence of the wild type gene. Six slow growing Tyr⁺ mutants were directly sequenced and the *tas* gene in these mutants was also found to have the wild type sequence. When *tas* was cloned into a lower copy number vector, pACYC184, and introduced into WU3610_(old) it allowed a slow rate of growth on minimal plates lacking tyrosine. However, the growth rate was still greater than that observed for the slow growing Tyr⁺ mutants. Colonies were visible in two days rather than the six to seven days for the starvation-associated mutants and compared to the one to two days when *tas* was on the high copy number vector pGEM-3Zf(-). The efficiency of complementation of the tyrosine requirement was presumably associated with the relative copy number of the two plasmids.

The region we sequenced was originally mapped to 64 minutes on the *E.coli* genetic map with the aid of a 89 base pair homology to a sequence (Accession number L14681) previously identified (Jackowski *et al.*, 1994). Recently the sequence for the entire *E.coli* chromosome has become available and the *tas* fragment was unambiguously located to 2978.4 kb on the *E.coli* physical map. ORF II could also be identified and would appear to be the 3' portion of the gene *ygeD* with its associated promoter lying beyond the boundary of the cloned *tas* fragment. There would appear to be a single base pair difference between our *tas* coding sequence, obtained from a B/r strain, and the database sequence obtained from *E.coli* K-12. This base change does not result in an amino acid change and so the Tas proteins in *E.coli* B/r and K-12 are identical.

The multicopy *tas* plasmid, pART34, totally failed to complement a range of other auxotrophies due to ochre mutations, including *hisG4* and *argE3* in AB1157 and *trpE65* in WP2. *tas* was therefore unlikely to be involved in general ochre suppression. Neither was the plasmid able to complement a phenylalanine auxotrophy in the strains N3078 (*pheA13::Tn10*) or KA12 (Δ *pheA-tyrA-aroF*). This suggested a very specific role for *tas* in complementation of the defective tyrosine biosynthetic capability in WU3610_(old). Examination of the terminal pathways for the aromatic amino acids tyrosine and phenylalanine Figure 3.22.2 show that they are very similar. An initial common pathway terminates at chorismate where the terminal pathways for tyrosine and phenylalanine branch.

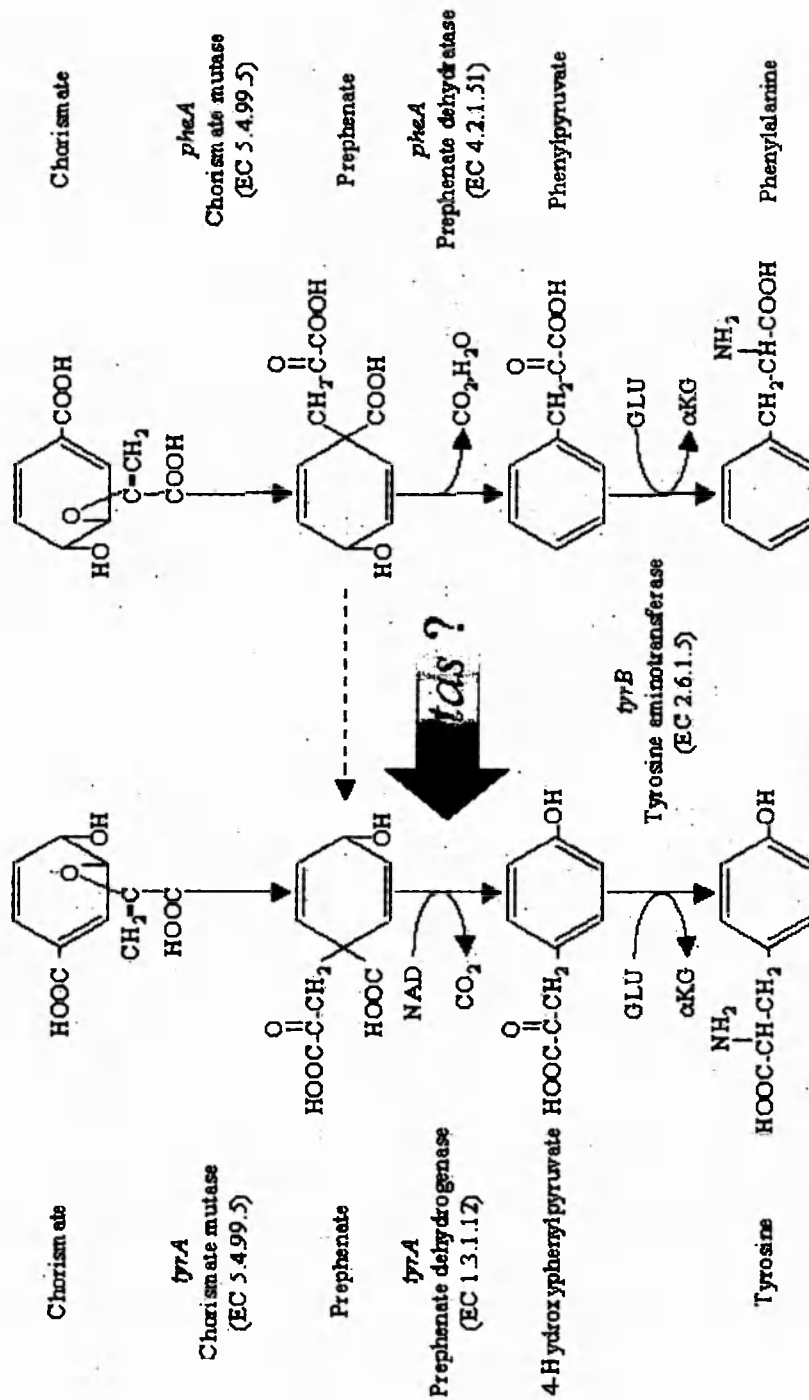
Biosynthesis proceeds via bifunctional enzymes with chorismate mutase (CM) and prephenate dehydrogenase activity in TyrA (T-protein) or an alternate CM and prephenate dehydratase activities in PheA (P-protein). The Tas protein sequence showed significant homology to several members of the aldo-keto reductase superfamily of enzymes which catalyse reactions involving reduction of carbonyl groups. The prephenate dehydrogenase step in tyrosine biosynthesis would appear to involve a similar step and could be complemented by Tas if the necessary chorismate mutase activity was also present in the cell. WU3610 contains a fully functional P-protein, with associated CM activity, available to complement or supplement the dysfunctional (or partially functional) CM activity of the T-protein (see below). The tyrosine auxotrophy in this strain would therefore appear to be due to a specific lack of prephenate dehydrogenase activity.

Terminal tyrosine and phenylalanine biosynthetic pathways

The terminal pathways of tyrosine and phenylalanine biosynthesis are very similar (as illustrated) with the final products differing by a single hydroxyl group on the aromatic ring. Both pathways start with chorismate and involve bifunctional enzymes; TyrA-chorismate mutase/prephenate dehydrogenase and PheA-chorismate mutase/prephenate dehydratase. In both cases, the terminal amino transferase reaction is performed by the product of the *tyrB* gene. In *tyrA14* mutants, a suppressor gene *tas* has been found that can specifically complement the absence of prephenate dehydrogenase activity (it is unknown whether NAD is still required as a cofactor). Chorismate mutase activity is supplied by the functional PheA protein creating prephenate (dotted red arrow), which can enter both the pathways leading to phenylalanine and the pathway, mediated by *tas* (large red arrow), leading to tyrosine.

Figure 3.22.2

Terminal tyrosine and phenylalanine Biosynthetic pathways



The two-plasmid system of Kast *et al.* (1996) was used to demonstrate a specific role for Tas in complementing the tyrosine auxotrophy of WU3610. The *E.coli* strain KA12 carries a deletion of both the bifunctional *tyrA* and *pheA* genes, consequently there is no source of endogenous CM activity from either the T-protein or the P-protein. In the two-plasmid system, when *tas* replaced the mono-functional prephenate dehydrogenase (*Erwinia herbicola tyrA*) gene on pKIMP-UAUC, it provided full complementation of tyrosine auxotrophy in WU3610. In KA12 when CM activity was also provided, either *tyrA* or *tas* allowed the strain to grow on minimal media lacking tyrosine Table 3.22.2. In WU3610, overexpression of CM activity had no effect on the ability of the strain to grow on minimal media but *tas* alone was sufficient to restore growth.

Studies with the *pheA13::Tn10 tyrA* double mutant strain (CM1338) showed that the *tyrA14* mutation may retain some chorismate mutase activity. The *tas* gene was still able to complement the tyrosine auxotrophy in the double mutant strain but since *tas* complementation required a functional CM activity either the *pheA13::Tn10* allele retains this activity or the *tyrA14* allele, which is assumed to be unchanged by the transduction, provides the necessary function.

In an attempt to discover the normal role of *tas*, a deletion strain (CM1355) was constructed. Growth of CM1355 was indistinguishable from that of its parent WU3610_(old), when streaked to either L-agar or minimal medium containing tyrosine and leucine. Clearly, *tas* is not an essential gene for growth.

Table 3.22.2

Complementation of amino acid auxotrophies

Strain	Plasmids	Media					
		MTP	MT	MP	MLT	ML	M
KA12	pKCMT-W	+	-	-	ND	ND	-
	pKIMP-UAUC	+	-	-	ND	ND	-
	pART34	+	-	-	ND	ND	-
	pART51	+	-	-	ND	ND	-
	pART52	+	-	-	ND	ND	-
	pKIMP-UAUC						
	pKCMT-W	+	+	+	ND	ND	+
	pKIMP-UAUC						
	pART34	+	-	-	ND	ND	-
	pKCMT-W						
	pART51	+	-	+	ND	ND	-
	pKCMT-W						
	pART52	+	+	+	ND	ND	+
WU3610	pKCMT	ND	ND	ND	+	-	-
	pKIMP-UAUC	ND	ND	ND	+	+	-
	pART51	ND	ND	ND	+	+	-
	pART52	ND	ND	ND	+	+	-

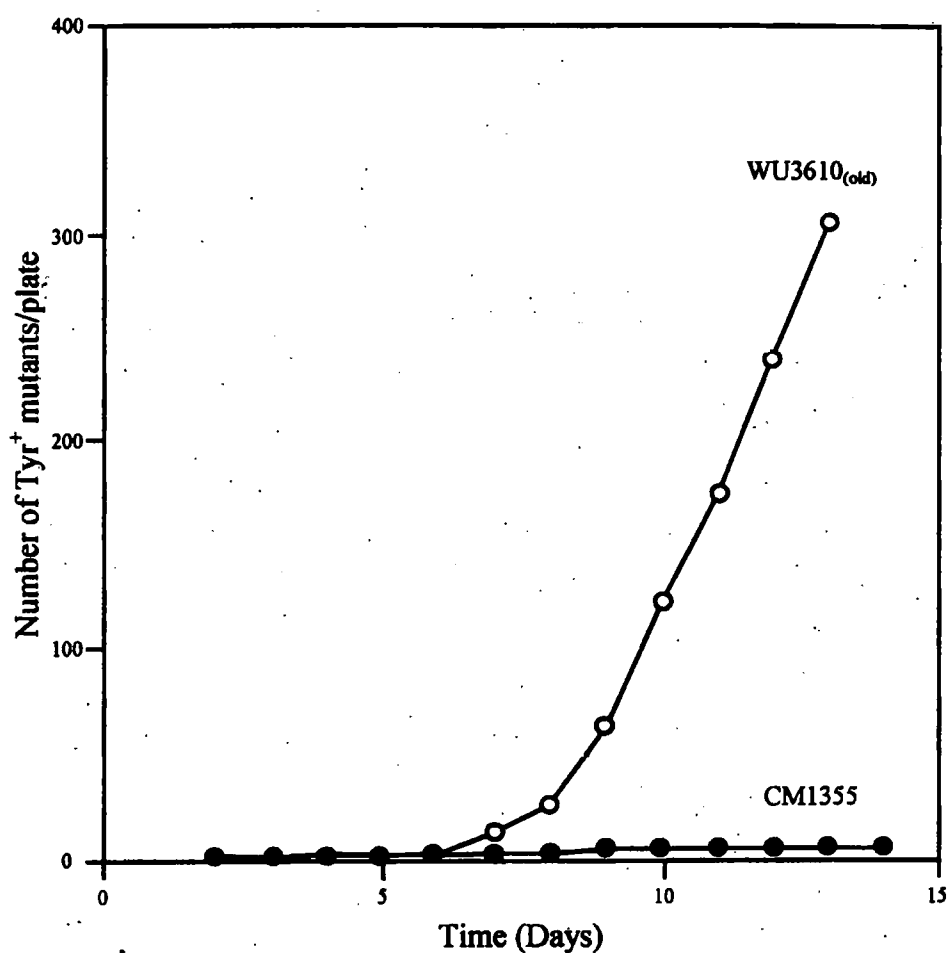
Table indicates the results from suppression studies using plasmids carrying monofunctional derivatives of enzymes involved in the terminal pathways of phenylalanine and tyrosine biosynthesis. Strain KA12 carries a deletion through the genes coding for TyrA and PheA. WU3610 carries the *tyrA14* mutation. (M) Is minimal salts agar, (L) leucine, (P) phenylalanine and (T) tyrosine were added at 10µg/ml. ND indicates test not applicable to that strain. + Indicates suppression of phenylalanine, tyrosine or combined auxotrophy. - Indicates no suppression, strains did not grow on minimal agar plates lacking the relevant amino acid.

When the strain was tested for the ability to show mutation under conditions of tyrosine starvation, there was a very pronounced difference. CM1355 did not show any starvation-associated mutation under the conditions normally used for WU3610. Further experiments were performed in which starvation-associated mutation was examined in *mutL::Tn10* derivatives of WU3610_(old) (CM1421) and CM1355 (CM1422). Results are shown in Figure 3.22.4. Again, Δ *tas* completely abolishes the appearance of slow growing tyrosine independent mutants despite an increase in the rate of appearance and number of colonies in the *tas*⁺ strain. These results are entirely consistent with those obtained by Bridges (1995a) and Bridges *et al.* (1996) where the effects of *mutY* and *mutM* mutators in WU3610 were shown to be broadly similar to the results presented above. Unfortunately neither *mutY* or *mutM* derivatives of the Δ *tas* strain were examined in this case although there is no reason to believe they would behave any differently to the *mutL::Tn10* derivative. These results suggest three possible reasons for the observed effect of *tas*: i) Δ *tas* strains lose viability faster than parental strains, ii) Tas protein is necessary for the mutation process, iii) *tas* is the target gene for stationary phase mutation in this system.

A viability experiment was performed in which CM1355 and WU3610_(old) were plated on minimal media lacking tyrosine at two cell densities, 3×10^8 and 1×10^6 per plate. Results for the high cell density plating are shown in Figure 3.22.5. At high cell densities both strains behaved similarly; there was no increase in viable count over the first two days, after which viability declined slowly.

Figure 3.22.3

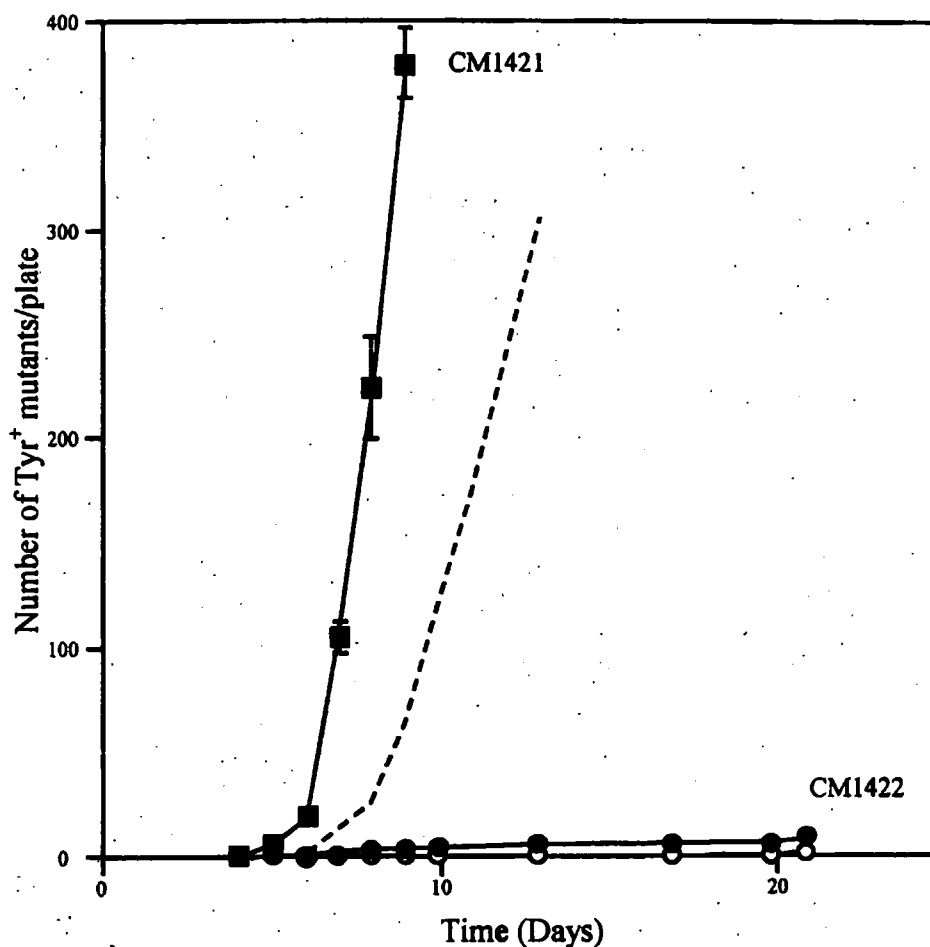
Starvation-associated mutation in a *tas*⁺ and *tas*⁻ strain



A representative starvation-associated mutation experiment showing the appearance of slow-growing mutants on glucose minimal salts plates containing leucine at 27°C. WU3610_(old) (○) is the parental *tas*⁺ strain and CM1355 (●) contains the $\Delta tas1::Km^R$ allele. 3×10^8 bacteria were plated per plate and incubated at 27°C.

Figure 3.22.4

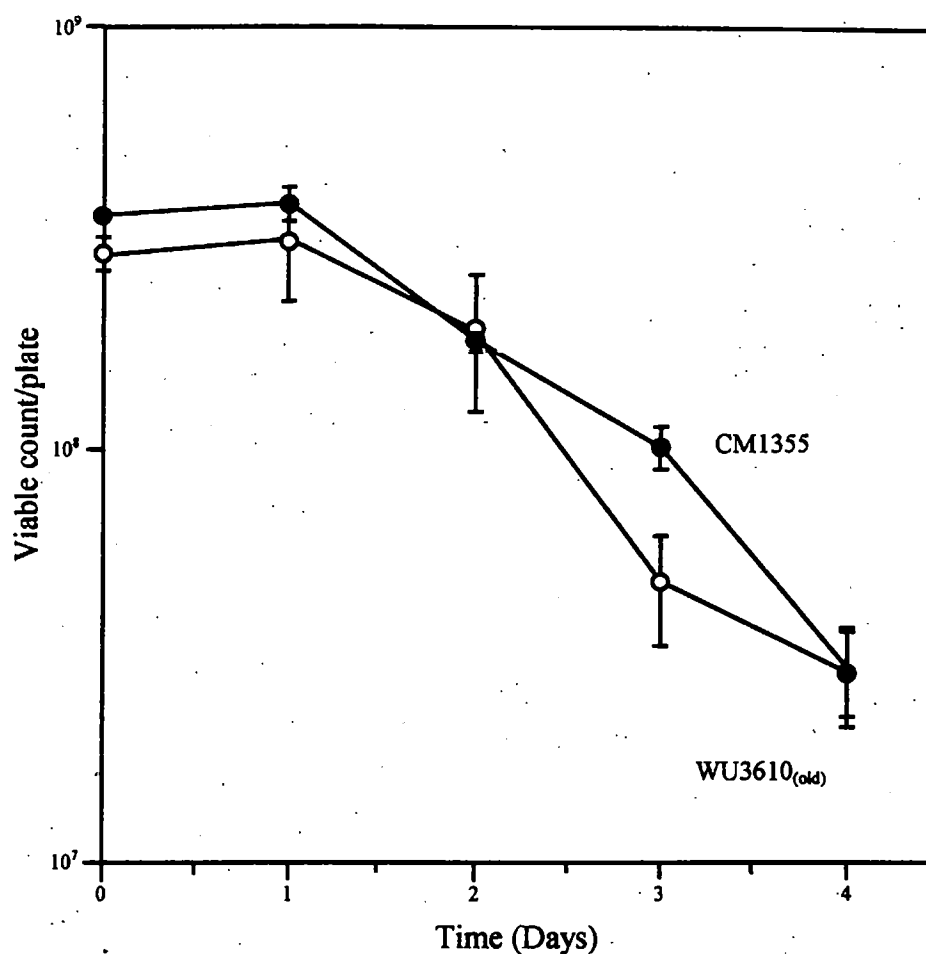
**Starvation-associated mutation in a *mutL* derivative of WU3610_(old) (CM1421)
and a *mutL* Δ *tas* strain CM1422**



Starvation-associated mutation experiment with a *mutL* derivative of the *tas*⁺ strain WU3610_(old) (CM1421, ■) and a *mutL* derivative of the Δ *tas* strain CM1355 (CM1422, ●). CM1355 (○) is included for reference. Dotted line is taken from Figure 3.22.3 for comparison with WU3610_(old). The data points are the means of three independent experiments performed on minimal salts agar plus leucine at 27°C.

Figure 3.22.5

**Viability of WU3610_(old) and its Δ *tas* derivative, CM1355
when plated at high cell density on minimal medium lacking tyrosine**



Viability determination of WU3610_(old) (○) and its Δ *tas* derivative CM1355 (●) when starved on agar plates for the amino acid tyrosine. About 3×10^8 bacteria were plated at 27°C and experiments were performed using the whole plate wash off procedure. Points are the mean of three experiments.

If the curves are extrapolated there may be a faster decline in the Δ *tas* strain after three to four days, although viability beyond this has not been tested. It was observed

that when WU3610 was plated at very low density (about 200 cfu per plate) onto minimal agar lacking tyrosine and incubated at 27°C, very small colonies were observable after about 18-20 days, no colonies were visible in the Δtas strain. Nearly all of the WU3610 cfu plated eventually formed colonies so viability was essentially 100%. This result suggests i) that ability to grow on media lacking tyrosine is intrinsic to WU3610 even when a limited number of chromosomal copies of *tas* are present, ii) that inviability of this strain seems to be associated with plating density as at very low density WU3610 maintains complete viability for at least 18 days and iii) that *tas* is absolutely required either for long term survival of the cell, for *de novo* tyrosine synthesis enabling growth or for utilisation of a trace nutrient in the agar allowing growth.

If the following assumptions are made i) that the same *tas* dependent process is responsible for growth in wild-type WU3610 and the slow growing Tyr⁺ starvation-associated mutants and ii) that the relative growth rate is indicated by the time at which colonies become visible and is a linear reflection of protein level in the cell then, there is approximately a three fold difference between Tas levels in the wild-type cell and the slow growing mutants. Therefore, the lack of stationary phase mutation in CM1355 cannot simply be explained purely by differential viability between the two strains, at least over the short term.

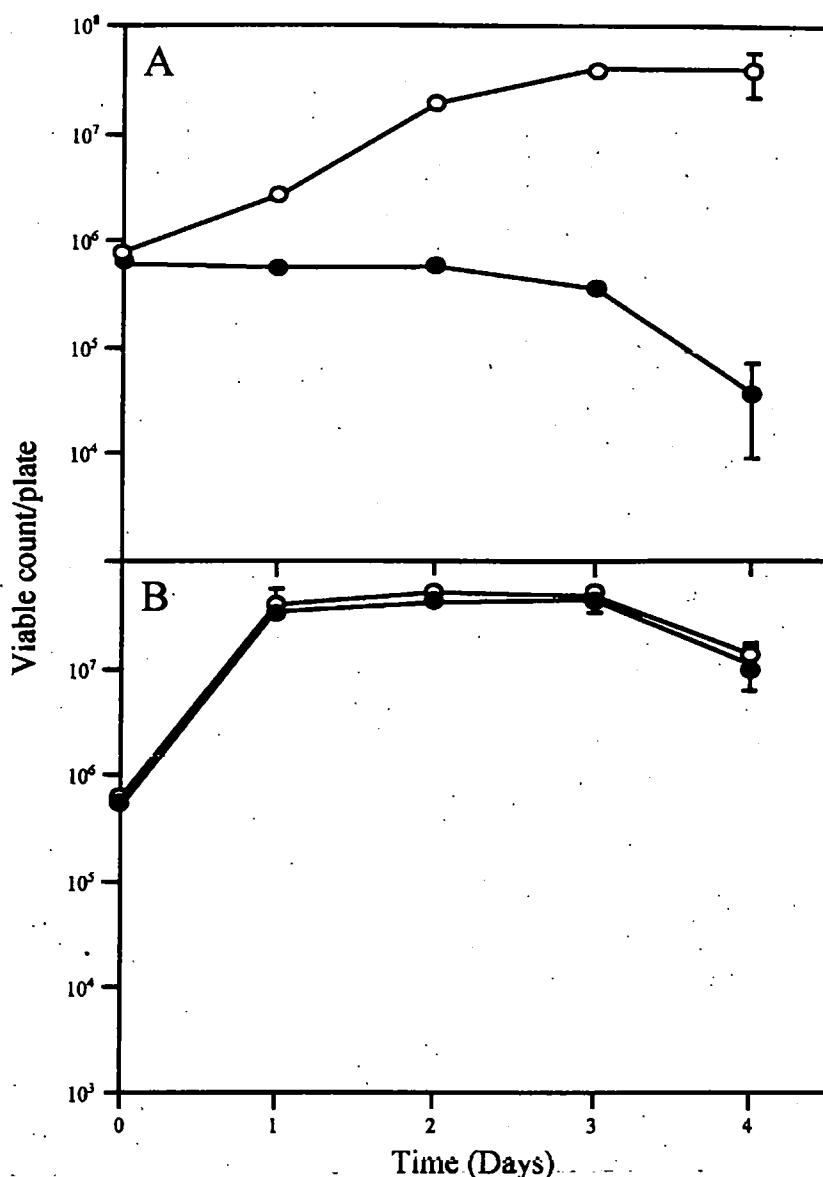
When plated at low cell density, WU3610_(old) increased in viable count to a plateau of about $6-7 \times 10^7$ per plate. However, CM1355 did not show any increase in bacterial count when starved for tyrosine although residual growth at low cell density

was similar to that of WU3610_(old) when starvation was for leucine **Figure 3.22.6**. These results provide further evidence that the activity of *tas* is required for residual growth at low cell density in the absence of tyrosine. It also suggests that the ability to undergo a very small amount of growth may be necessary if starvation-associated mutation is to occur at the higher cell density normally used. One obvious conclusion is that *tas* confers the potential for a small amount of growth to occur in the absence of tyrosine, however, the role of *tas* may not be quite this simple. Slow growing revertants were absent even when plates were spiked with 0.1µg/ml of tyrosine to stimulate some residual growth or, when growth conditions were used as described previously (Bridges, 1994).

Therefore the final hypothesis, namely that *tas* may be the target gene for mutation must be considered. Since no alteration in the sequence of *tas* or its promoter region could be detected, in the slow growing Tyr⁺ revertants, one possibility was that the *tas* gene was amplified in these mutants. This would be entirely consistent with the observation that *tas* on a multicopy plasmid confers a Tyr⁺ phenotype. However, probing of DNA from six slow growing tyrosine independent mutants and two fast growing revertants revealed no sign of amplification of the *tas* region compared to a control region (*rpoB*) **Figure 3.22.7**. Experimental evidence has thus far provided no clue to the process whereby *tas* can result in Tyr⁺ colonies. Possible speculative mechanisms are thus considered in a later section.

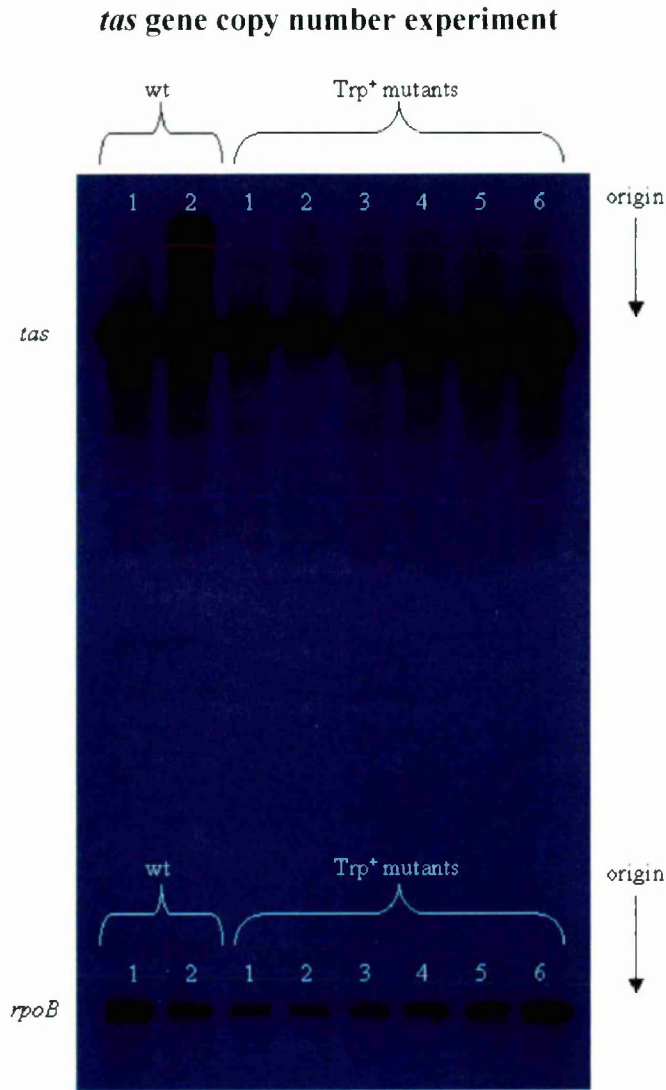
Figure 3.22.6

**Viability of *tas*⁺ and *tas*⁻ bacteria on leucine and on tyrosine starvation plates
when plated at low cell density**



Viability of WU3610_(old) (○) and CM1355 (●) under starvation conditions followed over 4 days. Plates were inoculated with about 10⁶ bacteria and viabilities were determined from whole plate wash-off experiments. Data points are the means of three or more independent experiments. (A) Shows viability of strains incubated on glucose minimal salts plates supplemented with leucine. (B) Shows the same strains on glucose minimal salts plates supplemented with tyrosine.

Figure 3.22.7



Blots of bacterial genomic DNA from two wild type and six independently isolated slow growing Trp⁺ mutants from starvation-associated mutagenesis experiments. The top tracks were probed with DNA complementary to the *tas* gene, while the bottom blot was probed with a fragment from *rpoB*. There is some variation between DNA concentrations for each sample, however the relative intensity of the bands, for the wild type and mutant lanes, was the same in both *tas* and *rpoB* blots. This shows that, within the confines of this system, *tas* did not show any amplification relative to an unrelated control sequence.

During an investigation into the effects of *rpoS* on the rate of starvation-associated mutagenesis it was observed that a *rpoS* mutant of WU3610_(new) (CM1376) showed a faster rate of appearance of the slow growing Tyr⁺ mutants than the *rpoS*⁺ parent strain **Figure 3.22.8** also see Appendix II. A Δ *tas* *rpoS* strain was constructed (CM1428) to see if this effect was *tas* dependent. In CM1428 as for the other *tas* deletion strains CM1355 and CM1415 starvation-associated mutation was totally abolished **Figure 3.22.9**. Examination of the viabilities of WU3610_(new), CM1376 and CM1428 show that the *rpoS* strain behaves similarly to the parent up to day three although extrapolation of the viability for this strain after day three would appear to indicate a drastic decline thereafter **Figure 3.22.9**. CM1428 shows an immediate decline in viability from day one, of about 60%, and then a plateau to day 3 whence the viability appears to mirror the decline in the *rpoS* single strain. While Δ *tas* abolishes starvation-associated mutation in WU3610 it would also appear to have an effect on viability over the longer term which is most pronounced when combined with a *rpoS* mutation.

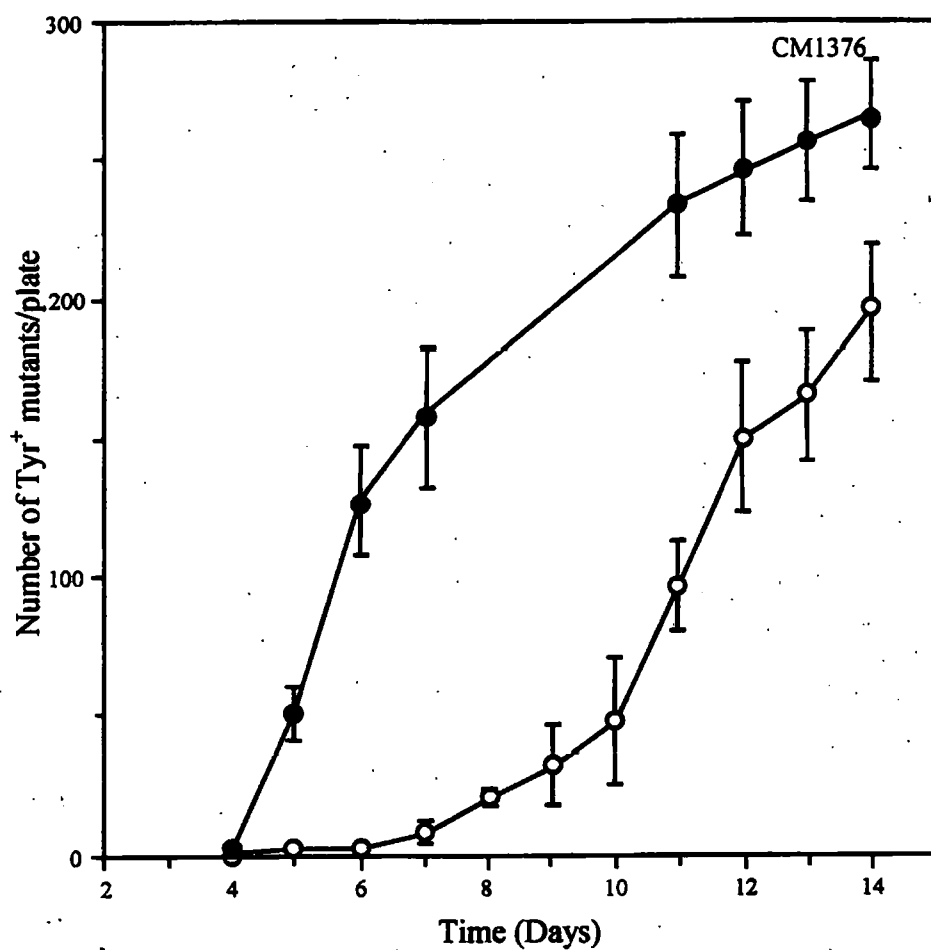
3.23 Discussion

The results presented have described a new gene *tas* which, when present on a multicopy plasmid, is able to suppress the tyrosine requirement of the *tyrA14* ochre strain WU3610. When a *tas* deletion is present (Δ *tas*), it prevents the appearance of the slow growing tyrosine-independent mutants that normally arise in this strain during prolonged incubation on minimal plates lacking tyrosine.

Figure 3.22.8

Starvation-associated mutation in WU3610_(new)

and its *rpoS* derivative CM1376



Starvation-associated mutation experiment with the *tas*⁺ strain WU3610_(new) (○) and its *rpoS* derivative (CM1376, ●). The data points are the means of three independent experiments performed on minimal salts agar plus leucine at 27°C.

Starvation-associated mutation and viability of *rpoS* and *rpoS* Δ *tas*

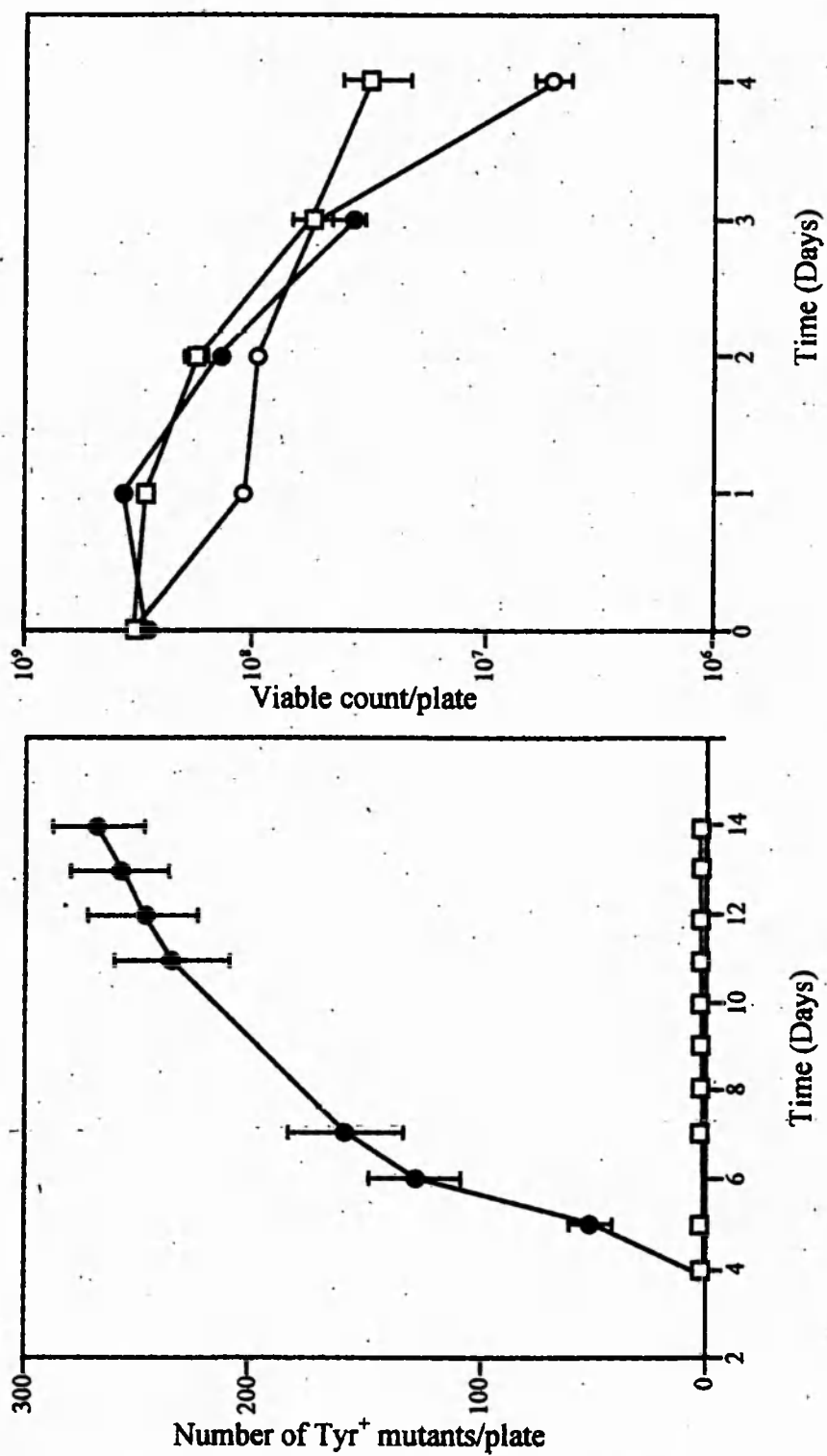
derivatives Of WU3610_(new)

Starvation-associated mutation in the *rpoS* (CM1376, ●), *Δtas* (CM1415, Δ) and *rpoS* *Δtas* (CM1428, ○) derivatives of WU3610_(new). About 3×10^8 bacteria were plated to minimal salts plates plus leucine and incubated at 27°C, points are the means of at least three experiments.

Viable determinations of WU3610_(new) (□), CM1376 (●) and CM1428 (○). Experiments were performed by using the whole plate wash-off procedure, points are the means of three experiments. Viability for CM1376 was followed for only three days because of the fast arising starvation-associated mutants.

Figure 3.22.9

Starvation-associated mutation and viability of *rpoS* and *rpoS* Δ tas derivatives Of WU3610_(new)



Δ *tas* also prevents the appearance of slow growing tyrosine independent colonies in strains lacking a component of MMR, despite an enhanced level of starvation-associated mutagenesis in *tas*⁺ strains.

The T-protein (product of the *tyrA* gene) is a bifunctional enzyme that catalyses two steps in the tyrosine biosynthetic pathway *i.e.* the chorismate mutase and prephenate dehydrogenase reactions **Figure 3.22.2** (Koch *et al.*, 1971a; 1971b; Sampathkumar and Morrison, 1982). Monofunctional derivatives of the T-protein have been produced (Dayan and Sprinson, 1971; Rood *et al.*, 1982) showing that the chorismate mutase function can operate without concomitant prephenate dehydrogenase activity. The tyrosine auxotrophy conferred by the *tyrA14* allele is the result of an ochre termination codon corresponding to amino acid residue 161 within the T-protein (Li *et al.*, 1991). Some evidence has been presented to show that the product of the *tyrA14* allele in WU3610 may retain chorismate mutase activity but not the prephenate dehydrogenase activity characteristic of the wild-type product. Since the chorismate mutase activity has been shown to reside in the N-terminal portion of the protein (Hudson and Davidson, 1984), the region of the gene translated could specify an active mono-functional protein. Alternatively, chorismate mutase activity may be complemented *in-trans* by the fully functional P-protein in WU3610. Whether or not one or both of these possibilities operate, it is clear that *tas* on a multicopy plasmid can complement the prephenate dehydrogenase deficiency of WU3610.

A database search revealed that Tas had a 35.5% amino acid identity with a putative aldo-keto reductase from the protozoan haemoparasite *Babesia bovis*. It also

displayed limited homology in the region of the active site with, among others, the genes for mouse aldose reductase, rabbit aldo-keto reductase and human alcohol dehydrogenase, which are all members of the aldo-keto reductase superfamily. Conservation of certain residues at the active site is wide spread amongst aldo-keto reductases with very different substrate specificities.

In the Tas protein, there are three regions where the conservation of sequence is particularly marked, residues 121-134, 177-180 and 228-238. When these regions are aligned with the equivalent sequences in the four proteins mentioned above the homology is immediately obvious Table 3.23.1. Based on this conservation of residues, Tas is probably a member of the aldo-keto reductase superfamily. The step in tyrosine biosynthesis catalysed by prephenate dehydrogenase has similarity to a carbonyl reductase reaction. If Tas does have carbonyl reductase activity, it is not unreasonable to assume that the protein has some small ability to act as a dehydrogenase on prephenate and thus complement the defect in *tyrA*. However, the normal function of the gene remains unknown, all that can be said is that it is not essential under normal circumstances.

Data presented show that *tas*⁺ bacteria but not those carrying a deletion in *tas* exhibit growth on minimal agar at low cell density. When WU3610 is plated at 10⁶ bacteria per plate, growth occurs but viability levels off at about 6-7 × 10⁷ cells per plate Figure 3.22.6. The plateau occurs whether starvation is for tyrosine or leucine and appears to be a limitation of bacterial density rather than exhaustion of a specific nutritional requirement.

Table 3.23.1

Homologies between Tas and aldo-keto reductases

Organism	Protein	Amino acid sequence ^a	
<i>E.coli</i>	Tas	121	134
		L Q T D Y L D L Y Q V H W P	
<i>B.bovis</i>	Aldo-keto reductase	42	35
		L N T D Y I D L L Q L H W P	
Mouse	Aldose reductase	100	113
		L K L D Y L D L Y L V H W P	
Rat	Aldo-keto reductase	101	114
		L Q L D Y V D L Y I I H F P	
Human	Alcohol dehydrogenase	106	119
		L Q L E Y L D L Y L M H W P	
	Consensus	L * D Y * D L Y * H W P	
<i>E.coli</i>	Tas	177	180
		G V S N	
<i>B.bovis</i>	Aldo-keto reductase	105	108
		G L S N	
Mouse	Aldose reductase	158	161
		G I S N	
Rat	Aldo-keto reductase	164	167
		G V S N	
Human	Alcohol dehydrogenase	159	162
		G L S N	
	Consensus	G * S N	
<i>E.coli</i>	Tas	228	238
		V E L L A Y S C L G F	
<i>B.bovis</i>	Aldo-keto reductase	159	169
		I A I L A Y A P L A G	
Mouse	Aldose reductase	205	215
		I A V T A Y S P L G S	
Rat	Aldo-keto reductase	211	221
		I V L V A Y S A L G S	
Human	Alcohol dehydrogenase	204	214
		L E V T A Y S P L G S	
	Consensus	* * A Y S * L G	

^a flanking numbers refer to the position of residues within the proteins.

Table shows homology between Tas and several other aldo-keto reductase enzymes. Amino acid sequences are listed, numbers flanking the sequence indicates position of the polypeptide within the relevant protein. Where four out of the five sequences contained the same residue at a particular site this is denoted as a consensus sequence. * Indicates a conservative amino acid substitution at that particular position.

A possibly similar effect was observed in continuous culture experiments performed by Munson and Bridges (1964). These experiments, involving the strain WP2 grown in minimal medium supplemented with tryptophan, demonstrated that occasionally the cultures were subject to "take-over". In this process a rare mutation occurred allowing prototrophs to colonise the wall of the culture vessel. While the culture vessel (wall area 1200mm²) could support a maximum of 4×10^9 bacteria if they were present in a close packed single layer, the greatest number observed never exceeded 1% of this value (4×10^7) (Munson and Bridges, 1964). The equivalent figure for an agar plate (area 5944mm²) gives a maximum bacterial density of 2×10^8 per plate ($(5944/1200 \text{ mm}^2) \times 4 \times 10^7$) which is within a factor of three of that observed. Whether this effect is due to contact inhibition or to some soluble signal molecule is uncertain but it is difficult to see how contact can be involved at the cell densities observed on minimal plates during starvation-associated mutation experiments.

It has been observed that derivatives of homoserine lactone are excreted into the growth medium by some bacterial species, e.g. *Pseudomonas aeruginosa*, *Aeromonas hydrophila* and *Vibrio anguillarum*, when the population approaches saturation. These compounds serve as indicators of population density (quorum-sensing autoinducers) and consequently lead to a reduction in population growth rate. There is some evidence that *Escherichia coli* cultures are also sensitive to cell density (Sitnikov *et al.*, 1996; Lange and Hengge-Aronis 1994). However, a similar intercellular signal molecule has yet to be found, although derivatives of homoserine lactone have been shown to act as intracellular signalling molecules increasing *rpoS*

transcription in *E.coli* cultures entering stationary phase (Huisman and Kolter, 1994). Another possibility is that cAMP could serve as a signalling molecule, the cell excretes 99.9% of its cAMP, production of which is increased in starving compared to growing cells (Buettner *et al.*, 1973; Matin and Matin, 1982). Alternatively, *E.coli* could have some, yet unidentified, signal molecule that it releases as it approaches saturation and is the analogue of quorum-sensing molecules found in other eubacteria.

Another explanation for the plateau effect may be that there are trace nutrients in the minimal agar that become limiting as the cells increase in density. In the case of tyrosine starvation, utilisation of this trace substance would require that *tas* is present. The fast growth rate of WU3610 carrying *tas* on a multicopy plasmid would tend to argue against this putative function of *tas*. If *tas* is overexpressed and is no longer limiting for growth the trace substance would become the limiting factor, this does not appear to be the case. Plasmid bearing strains form healthy colonies (4-5mm diameter) on minimal leucine media and reach a much higher total population per plate than that shown by WU3610 when it is plated at low cell density. Some *de novo* synthesis of tyrosine must therefore be taking place. It is interesting that the same amount of growth of WU3610 occurs on leucine starvation plates, that is not blocked by *tas*, possibly there is a *tas* equivalent to allow leucine synthesis?

Previous work on starvation-associated mutation, in other bacterial systems, has indicated that there may be a requirement for a certain amount of leakiness in order for mutants to arise (Mittler and Lenski, 1992; Jayaraman, 1995). In the WU3610

system at high cell density, the number of viable bacteria on the plate does not increase. However, examination of the lawn after 2 to 3 weeks showed evidence of population turnover and increase in biomass (lawn growth) with numerous small microcolonies visible under the microscope (Bridges, 1994). If we assume that the leakiness conferred by *tas* occurs at high as well as at low cell density it is reasonable to postulate that this property is also the reason why *tas*⁺ but not *tas*⁻ bacteria show starvation-associated mutation. Nevertheless, even under conditions where leaky growth was promoted by the addition of a small amount of tyrosine, there was still no evidence of slow growing revertants in the strains carrying the *tas* deletion.

This ineluctably leads to the conclusion that *tas* may be required not only for the continued viability of the slow growing revertants but also for their formation and raises the possibility that *tas* is also the target gene for the mutational process. A simple point mutation in the *tas* coding sequence is excluded by the absence of any detectable sequence difference between *tas* in WU3610 and a number of slow growing Tyr⁺ mutants. An alternative, that the mutation responsible for the Tyr⁺ colonies is not a sequence change but an amplification of the *tas* region, has been considered and examined experimentally. Data obtained with a related strain show that deletions are prone to occur in starved cells (Bridges and Timms, 1997, and section 3.1 of this thesis). Therefore, other chromosomal rearrangements including duplications might also be expected to occur in nutritionally deprived cells.

Gene duplications are by no means uncommon in populations of *E.coli* cells (Anderson and Roth, 1977; 1978; Sonti and Roth, 1989). The population that we

plate on to minimal agar is thus likely to contain a proportion of cells with duplication of the *tas* region and there would be on the plate bacteria that had *tas* duplications at the time of plating and some in which the first duplication arose on the plate. These bacteria would be expected to have a selective growth advantage by virtue of the increased *tas* gene dosage perhaps enabling them to grow into the observed microcolonies. Within these microcolonies, further duplications could occur until a cell was produced capable of giving rise to a visible colony. Similar processes have been observed in other systems where gene amplification enables cells to overcome nutritional deprivation (Horiuchi *et al.*, 1963; Tlsty *et al.*, 1984; Sonti and Roth, 1989). Based on relative growth rate, a 3 to 4-fold amplification of the *tas* gene is all that would be required to provide the growth advantage observed in the Tyr⁺ mutants. Duplication was also found to be a result of SOS induction or introduction of constitutively activated *recA* (Dimpfl and Echols, 1989). This may be significant in view of the findings of Taddei *et al.* (1995; 1997a) that SOS is induced in old colonies, although whether this also applies to starving colonies is unknown. However, *tas* gene duplication could not be demonstrated in a number of slow growing Tyr⁺ revertants and so this explanation must be discarded at least for the *tas* gene itself.

Some form of mutagenesis would appear to be involved in this *tas* dependent process as strains defective in MMR or ⁰G show large increases in the number of starvation-associated Tyr⁺ mutants. The number of mutants that occur in the mismatch repair deficient strains suggests that the Tyr⁺ phenotype is due to a forward mutation that influences *tas* expression, perhaps a gene inactivation event. Typical forward

mutation systems can yield spontaneous mutant frequencies in the range 10^{-4} to 10^{-11} (Sparling and Blackman, 1973; Andr sson *et al.*, 1976; Timms and Bridges, 1993) depending upon the types of event and the number of sites where a mutation would result in a mutant phenotype. Therefore, 3×10^8 WU3610 cells could conceivably contain several hundred pre-existing Tyr⁺ mutants which would correspond well with the number of slow growing colonies observed during starvation experiments. Thus, a forward mutation could be sufficient to account for some or all of the Tyr⁺ colonies observed on minimal plates in mismatch proficient strains, although some mutants subsequently arising on the plate cannot be discounted. If the mutation is in a gene other than *tas* but nevertheless affects *tas* activity, there are two possible mechanisms that might be considered; i) the mutation may increase the transcription rate of *tas* and ii) post transcriptional factors affect (increase) Tas protein levels.

The first hypothesis is that a mutation increases transcription from *tas* allowing growth on minimal medium lacking tyrosine. What factors are likely to affect transcription levels from *tas*? The most obvious would be inactivation of a specific *tas* repressor protein much in the manner that inactivation of *lacI* leads to expression of the *lacZYA* operon. Identification of this protein would require identification of the upstream (or downstream) sequences responsible for interaction with the repressor. Subsequent isolation of the repressor by virtue of its interaction with this sequence would then be possible.

An alternative is that *tas* expression is affected by one or more of the global regulators that are recognised as central to the adaptation of *E.coli* under various

environmental conditions. These regulators include; the stationary phase sigma factor RpoS (σ^S), the histone like protein H-NS, the leucine responsive protein Lrp, the integration host factor IHF, the factor for inversion stimulation FIS and the cAMP-CRP receptor protein system. The effect of the RpoS sigma factor on WU3610 has been examined in a null mutant. It was found to increase the rate at which the Tyr⁺ mutants appear on minimal plates but it does not seem to affect the number of such mutants arising. Therefore, the defective RpoS appears to enhance the effect of an existing mutation but cannot be the primary cause of the Tyr⁺ mutants.

All the remaining proteins have been shown to be involved in DNA binding and possibly in chromosome organisation (Spassky *et al.*, 1984; Bertin *et al.*, 1990; Higgins *et al.*, 1990; Yoshida *et al.*, 1993b; Ussery *et al.*, 1994; Schneider *et al.*, 1997). CRP, FIS, H-NS, IHF and Lrp have specific binding sites or recognise specific DNA conformations (although in some cases with little apparent sequence specificity) and may result in bending of the DNA at these sites (Thompson and Landy, 1988; Hodges-Garcia *et al.*, 1989; Gille *et al.*, 1991; Kahn and Crothers, 1992; Wang and Calvo, 1993; Pan *et al.*, 1996; Spurio *et al.*, 1997). Local DNA bending may be a major determinant of the level of gene expression along with the relative level of supercoiling of DNA in the region of gene promoters. Since supercoiling of DNA is known to be important in regulating gene expression other proteins including DNA topoisomerase I and DNA gyrase could also potentially have an effect on *tas* expression. Another basic histone like protein HU also binds DNA with less apparent sequence specificity but this protein can also affects DNA structure and gene expression. While CRP, FIS, IHF and Lrp have been found to

both positively and negatively affect expression of specific genes (Schultz *et al.*, 1988; Newman *et al.*, 1992; Chuang *et al.*, 1993 Wang and Calvo, 1993; Altuvia *et al.*, 1994; Xu and Johnson, 1995a; 1995b; 1995c; 1997; González-Gil *et al.*, 1996), H-NS was found to inhibited expression in most, but not all, cases studied (Ueguchi *et al.*, 1993; Yoshida *et al.*, 1993a; 1993b; Bokal *et al.*, 1997; Donato *et al.*, 1997; Mukerji and Mahadevan, 1997; reviewed in Atlung and Ingmer, 1997). A mutation in any of these genes affecting chromosome structure could well have effects on the level of transcription from the *tas* gene. Interestingly some H-NS mutants have been found to have a slow growth rate (Barth *et al.*, 1995), which is consistent with the observation that the Tyr⁺ mutants used to generate the complementation libraries were also slow growing even in rich medium. It has also been observed that some genes in *hnsA rpoS* double mutants have transcription levels above that seen in *hnsA* or *rpoS* single mutants (Arnquist *et al.*, 1994; Barth *et al.*, 1995; Yamashino *et al.*, 1995). This is also consistent with the observation that introducing a *rpoS* deletion allele, into WU3610_(new), increases the rate at which the Tyr⁺ colonies start to appear (Appendix D).

The second possibility must be considered in relation to increased stability of the Tas protein or of the *tas* mRNA as well as increased translation. There is evidence that some proteins increase in stability as bacteria enter stationary phase. An example of one such protein is the stationary phase sigma factor (RpoS or σ^S) coded for by the *rpoS* gene. σ^S protein levels can increase four to six fold in starving *E.coli* despite an apparent decrease in *rpoS* transcription and translation (Zgurskaya *et al.*, 1997). In growing cells σ^S has a half-life of about 1.5 to 2.5 minutes (Lange and Hengge-

Aronis, 1994) but in stationary cells the half-life increases to more than 10 minutes, which would adequately account for the increased protein level. Subsequently Schweder *et al.* (1996) have shown that strains deficient in ClpX, which is a subunit of the ClpP protease, exhibit exponential phase σ^S levels equivalent to those in stationary cells. Deficiency in this one subunit would therefore appear to be responsible for the increase in σ^S protein levels. *E. coli* contains a number of other cytosolic protease's such as Fa, Lon, So and Ti as well as two other subunits of ClpP (ClpA and ClpB). Each enzyme has its own substrate specificity although the proteolytic activities of most overlap. Inactivation of any of these enzymes could affect the nature and rate of proteolysis and could conceivably increase Tas stability enough to allow slow growth on minimal media lacking tyrosine.

The rate of appearance of the Tyr⁺ slow growing mutants was observed to increase in strains defective in RpoS. Does this mean that functional σ^S may induce a protease responsible for increased proteolysis in stationary phase? It has been known for some time that starvation for a variety of required nutrients leads to an elevated rate of degradation of proteins that are stable in growing cells (Mandelstam, 1960; Pine, 1973). If protease induction by σ^S were responsible for this observation, a *rpoS* null allele might be expected to increase general protein stability in starving cells. In the slow growing Tyr⁺ mutants, which may already be deficient in one or more proteases the effect could be to further increase Tas stability in stationary phase and increase the observed rate at which Tyr⁺ colonies appeared.

Increased stability of the *tas* mRNA transcript could also contribute to an increase in

Tas protein levels. Transcript half lives of between 40 seconds and 20 minutes have been measured (Pedersen *et al.*, 1978) but the elements that contribute to stability of a particular mRNA are diverse and in some respects little understood. Undoubtedly primary and secondary structure of the transcript is very important with features such as 5' and 3' untranslated regions, hairpin formation and polyadenylation all playing a role in determining the overall stability of the transcript (Belasco *et al.*, 1985; 1986; Newbury *et al.*, 1987; Emory *et al.*, 1992; Alifano *et al.*, 1994; Xu and Cohen, 1995; Kushner, 1996; Carrier *et al.*, 1997). What is clear is that no single mechanism is responsible for regulating mRNA decay but that each transcript may be degraded by several different pathways the balance of which leads to its nominal relative stability. Since the increase in the number of mutants in strains deficient in mismatch repair suggests that there are genetic elements involved in the appearance of Tyr⁺ colonies the genes known to be involved in mRNA stability must be considered. The most obvious of these are the exoribonucleases; RNase II (*rnb*), PNPase (*pnp*) and RNase PH (*rph*) and the endoribonucleases; RNase I (*rna*), RNase III (*rnc*), RNase E (*rne/ams*) in the form of the RNA degradosome and RNase M. Most of the above enzymes have specific roles in mRNA decay, for example PNPase is the primary enzyme involved in degradation of poly(A) tails (O'Hara *et al.*, 1995) but usually one or more alternative RNases can compensate in null mutants. However, deficiency in one or more RNases could well lead to increase in the half-life of the *tas* transcript and lead to increased product synthesis. The above list is not intended to be exhaustive indeed *E.coli* may contain as yet unidentified degradative RNases as well as those enzymes involved in rRNA or tRNA maturation displaying unrecognised activities. Unfortunately, because the termini of the *tas* transcript have not been

elucidated experimentally no comment can be made about potential stability of the mRNA molecule or possible degradative pathways.

The above mechanisms must remain speculative at present because little is known about *tas* function or regulation. However, the number of genes available as mutational targets is quite substantial and any of them may affect expression from *tas*. What is certain is that if any of the above mechanisms operate in the WU3610 starvation system they will provide an interesting explanation for the observations made with this strain.

3.3 Multiple mutations in the *rpsL* gene of *Escherichia coli*

High level resistance to or dependence on the antibiotic streptomycin occurs as a result of mutations in the *rpsL* gene, which codes for the S12 ribosomal protein. When spontaneous streptomycin-dependent (Sm^{D}) mutants were isolated and sequenced there was a high proportion that, as well as containing the mutation conferring streptomycin dependence, also carried another intragenic or ancillary mutation within *rpsL* (Timms *et al.*, 1992). It was subsequently shown that the majority of Sm^{D} *rpsL* alleles that carry ancillary mutations have a small growth advantage over single site mutants (Timms and Bridges, 1993). However, this growth rate advantage of approximately 10 to 23% when measured in established Sm^{D} strains was insufficient to account for the high frequency at which ancillary mutations were recovered. It was possible that extreme selection occurred at some point during expression of streptomycin dependence allowing ancillary mutants

selectively to outgrow their single site progenitors. Plasmids, with *rpsL* under the control of an inducible promoter, have been used to examine the effects of single and ancillary mutants under conditions where the cell would contain mixed populations of wild type and streptomycin resistant ribosomes.

3.31 Methods

Culture methods

Bacterial strains and plasmids are shown in Table 3.31.1. P1 phage transductions were performed as previously described. The $\Delta(\textit{srlR-recA})306::\textit{Tn10}$ allele, from RW202, was transferred into the strains CM1242 and CM1253. Potential transductants were streaked onto L-agar plates containing streptomycin and half of the plate was irradiated with 10 Jm^{-2} UV. $\Delta\textit{recA}$ strains are extremely sensitive to UV and show no growth on the exposed part of the plate after irradiation. Growth experiments were carried out in L-broth containing kanamycin (uninduced) or kanamycin plus 1mM isopropyl β -D-thiogalactopyranoside (IPTG) (induced *rpsL* gene). Plating was on to L-agar with additions where appropriate. Growth experiments involving CM1292 and CM1293 were performed in identical medium but with the addition of streptomycin. Antibiotic concentrations were as previously described Table 2.1.

Table 3.31.1

Bacterial strains and plasmids

Strain	Relevant genotype	Source or derivation
WP2	<i>trpE65, sulA1, lon-11</i>	E.M. Witkin
RW202	as WP2, $\Delta(srIR-recA)::Tn10$	R. Woodgate
CM1242	as WP2, <i>rpsL832</i>	Timms and Bridges, (1993)
CM1253	as WP2, <i>rpsL852</i>	Timms and Bridges, (1993)
CM1292	as CM1242, $\Delta(srIR-recA)::Tn10$	this study P1 (RW202) \times CM1242
CM1293	as CM1253, $\Delta(srIR-recA)::Tn10$	this study P1 (RW202) \times CM1253
Plasmid	Relevant genotype	Source or derivation
pEH1	<i>lacUV5</i> promoter, Km^R , <i>lacI^A</i>	E. Hennessey
pART4	as pEH1, <i>rpsL⁺</i>	this study
pART6	as pEH1, <i>rpsL852</i>	this study
pART7	as pEH1, <i>rpsL832</i>	this study

Construction of pART4, pART6 and pART7

General plasmid and DNA manipulations were as previously described. Cultures of *E.coli* strains WP2, CM1242 and CM1253 were grown as previously described. CM1242 and CM1253 were grown in the presence of streptomycin. The PCR boiling steps were performed as previously described. The lysate was used to seed a PCR which was performed as previously described and contained 100pmoles of primers; SM289 (5'CCAGGAGCTATTCCATGGCAACAG3') and SM961 (5'GGCGCACGTTTTTCGAGAGCTACTT3'). A *Nco*I site introduced by the primer SM289, encompassing the initiation codon of *rpsL*, and a *Bam*HI site 138 bp 3' to the *rpsL* termination codon were utilised to clone the gene into identical sites in the plasmid pEH1 Figure 3.31.1.

Induction of pART4, pART6 and pART7 in RW202

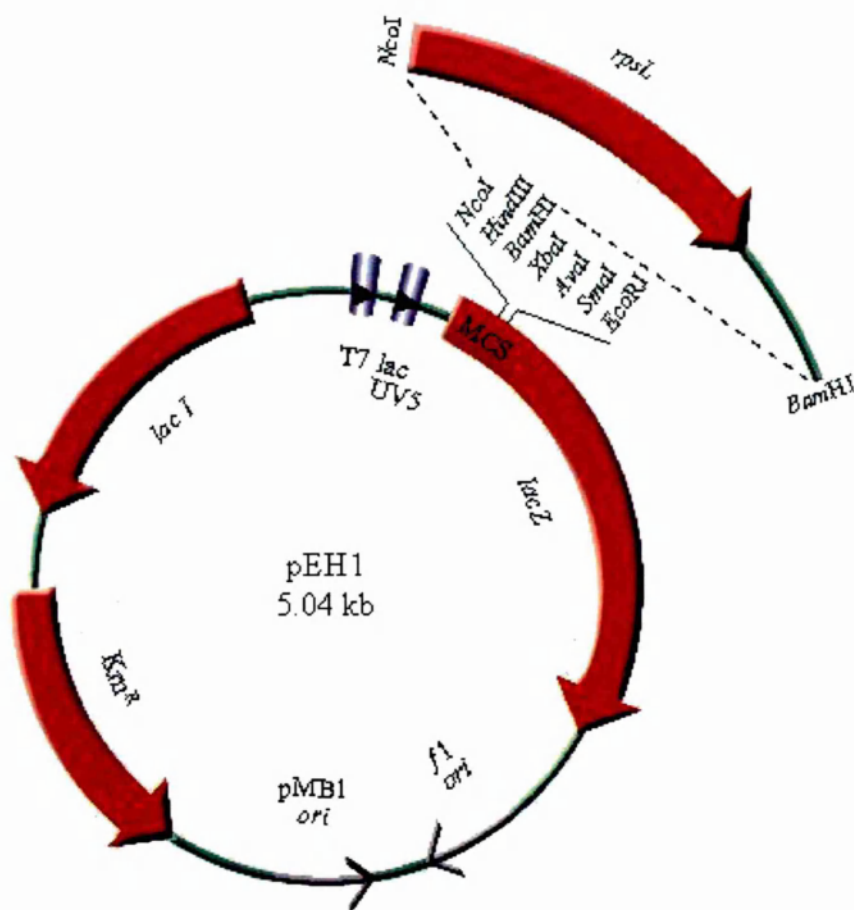
Approximately 10^4 bacteria from an overnight culture of RW202, containing one of the three plasmids, were used to inoculate 10ml of L-broth containing kanamycin and IPTG. Growth was followed for up to 12 hours by plating appropriate dilutions on to L-agar plates containing kanamycin. Plates were counted after 24 hours.

Growth of CM1292 and CM1293 containing pEH1 and pART4

Transformant colonies of CM1292 and CM1293 were emulsified in phage buffer. Because of the high spontaneous frequency of mutations to kanamycin resistance in

Figure 3.31.1

Plasmid pEH1



The structure of plasmid pEH1 is shown, including the inducible *lacUV5* promoter and the T7 promoter for *in vitro* RNA synthesis. The plasmid also carries a copy of the *lacI* repressor to increase repression of the uninduced *lacUV5* promoter. The *rpsL* gene was inserted into the *NcoI* and *BamHI* sites, in the multiple cloning site (MCS) of pEH1.

the two strains, plasmid DNA was extracted from the emulsified colony to confirm the presence of full sized pEH1 or pART4. However, true transformants were generally readily distinguishable by their larger colony size. Total counts were

performed and further dilutions were made such that approximately 10^4 cells were inoculated into 10ml of L-broth containing streptomycin and kanamycin. The growth rate was followed for up to 30 hours by plating appropriate dilutions onto L-agar plates containing kanamycin and streptomycin. The plates were incubated at 37°C and counted after 24 hours for CM1293pART4 and after 48 hours for CM1292pART4, CM1292pEH1 and CM1293pEH1 plates were incubated overnight before counting.

3.32 Results

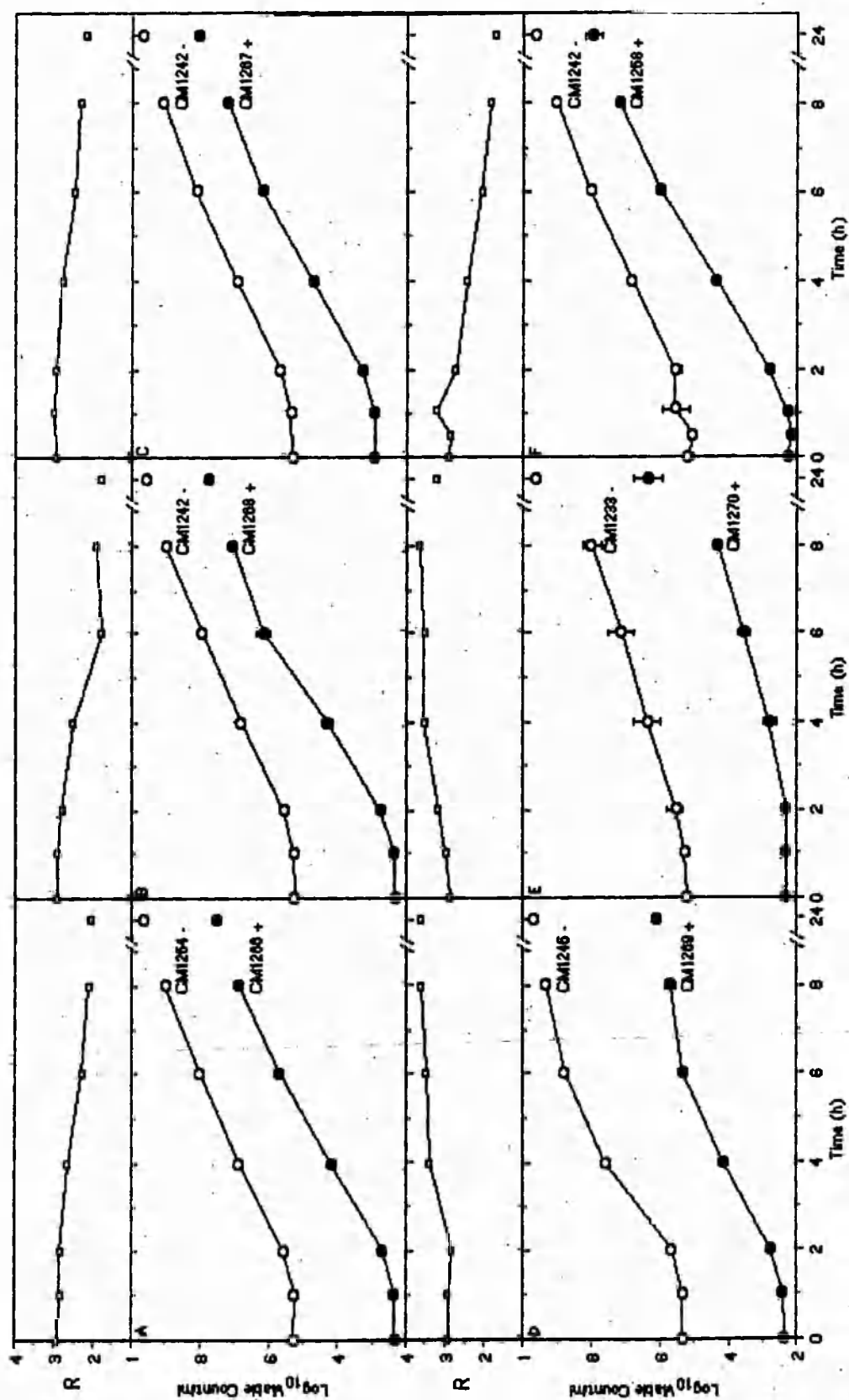
Sm^D mutants, as their name implies, require the presence of streptomycin in the medium for growth to occur. Thus, there could be severe negative selection for an expressed Sm^D mutant prior to challenge with streptomycin. Selection could also be severe if partially expressed Sm^D (or indeed streptomycin-resistant Sm^R) mutants with a high proportion of streptomycin-sensitive (Sm^S) wild type ribosomes were challenged with streptomycin. In order to examine the relative selective pressures under these conditions two strains; CM1242 containing *rpsL832* and CM1253 containing *rpsL852*, which is *rpsL832* plus an ancillary mutation, were selected for further examination. This pair not only displayed the greatest differential in growth rate between the single site and the ancillary mutant of the pairs examined Figure 3.32.1 but this ancillary mutation was also isolated repeatedly.

Growth of Sm^D mutant pairs with and without ancillary mutations

Growth of pairs of Sm^D mutants with and without ancillary mutations, incubated together in L-broth plus 1.2mg ml⁻¹ streptomycin from stationary phase inocula. Strains were spontaneous Sm^D mutants derived from WP2, + indicates non-suppressor tryptophan independent derivatives (●), - indicates original tryptophan requiring isolates (○). The tryptophan independent strain, usually the ancillary mutant, and its corresponding tryptophan requiring single site strain were mixed in a ratio of 1/1000 and grown with shaking at 37°C. □ = The ratio during the growth cycle of viable counts on minimal plates plus tryptophan (10µg ml⁻¹) to those on minimal plates lacking tryptophan, (R = log₁₀ viable count Trp⁻ bacteria – log₁₀ viable count Trp⁺ bacteria). CM1254, CM1268, CM1267, CM1269, CM1270 and CM1258 have ancillary mutations. Of particular interest to this study is section B showing the relative growth rates of CM1242 and the Trp⁺ derivative of CM1253 (CM1268) this pair shows the greatest growth advantage of the pairs examined.

Figure 3.32.1

Growth of Sm^D mutant pairs with and without ancillary mutations

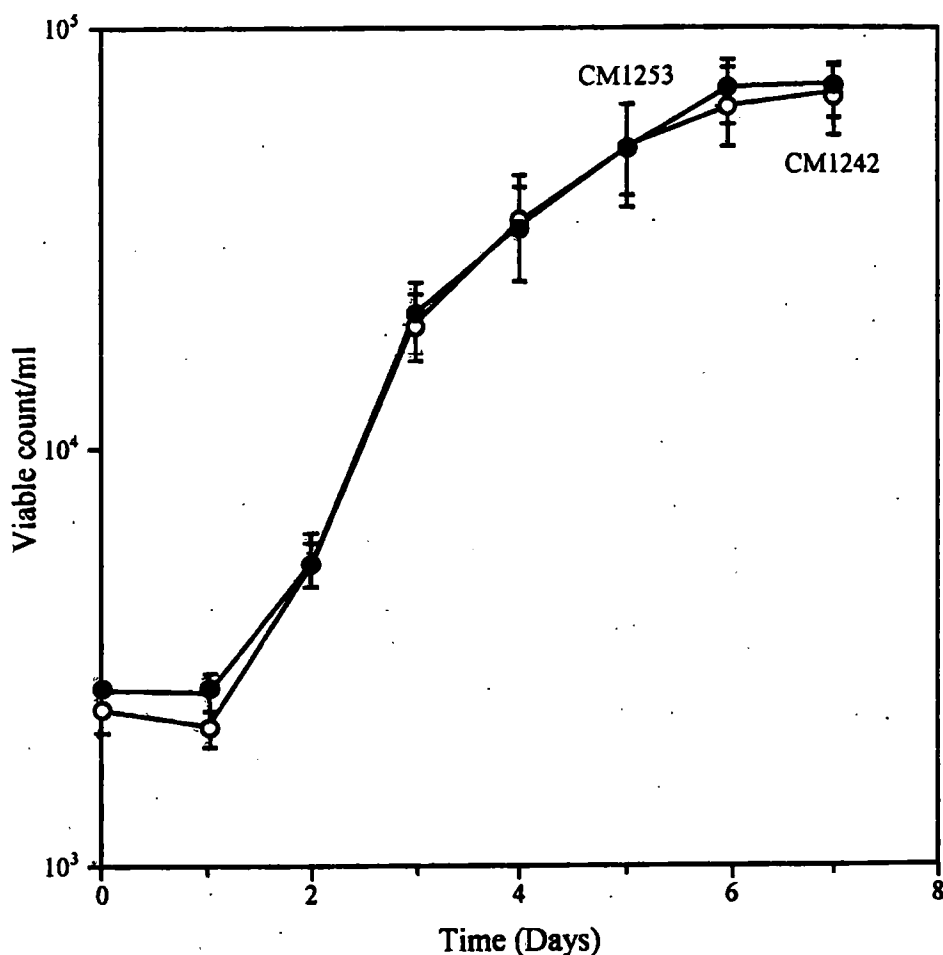


Once ribosomes have been exposed to streptomycin, they bind the drug very tightly. Even when streptomycin is withdrawn from the medium cells can go through several generations before growth ceases and viability eventually declines. The strains CM1242 and CM1253 undergo approximately five to six doublings, when grown initially at the standard streptomycin concentration used throughout this work, and both strains show almost identical growth curves under streptomycin deprivation conditions **Figure 3.32.2**. This makes the study of the effect of streptomycin deprivation on Sm^D strains very difficult once the antibiotic has been introduced into the system. To address this problem *rpsL832* and *rpsL852* as well as wild type *rpsL* were placed under the control of an inducible promoter on a plasmid. When induced, the Sm^D or wild type protein is produced without the need for prior maintenance of the strain in media containing streptomycin.

Growth experiments in L-broth in the presence or absence of 1mM IPTG were performed on RW202 containing each of the three plasmids. No significant changes in growth rate were observed among the three strains in the uninduced state compared to the host strain RW202 **Figure 3.32.3**. However, on induction with IPTG differences in growth rate became apparent. The divergence was most pronounced when the cultures were induced directly upon subculturing from a stationary overnight culture. Indeed, induction of the plasmid borne *rpsL* genes between one and two hours after subculturing, when the host had entered logarithmic growth phase, had little effect on the growth rate over the short term (data not shown).

Figure 3.32.2

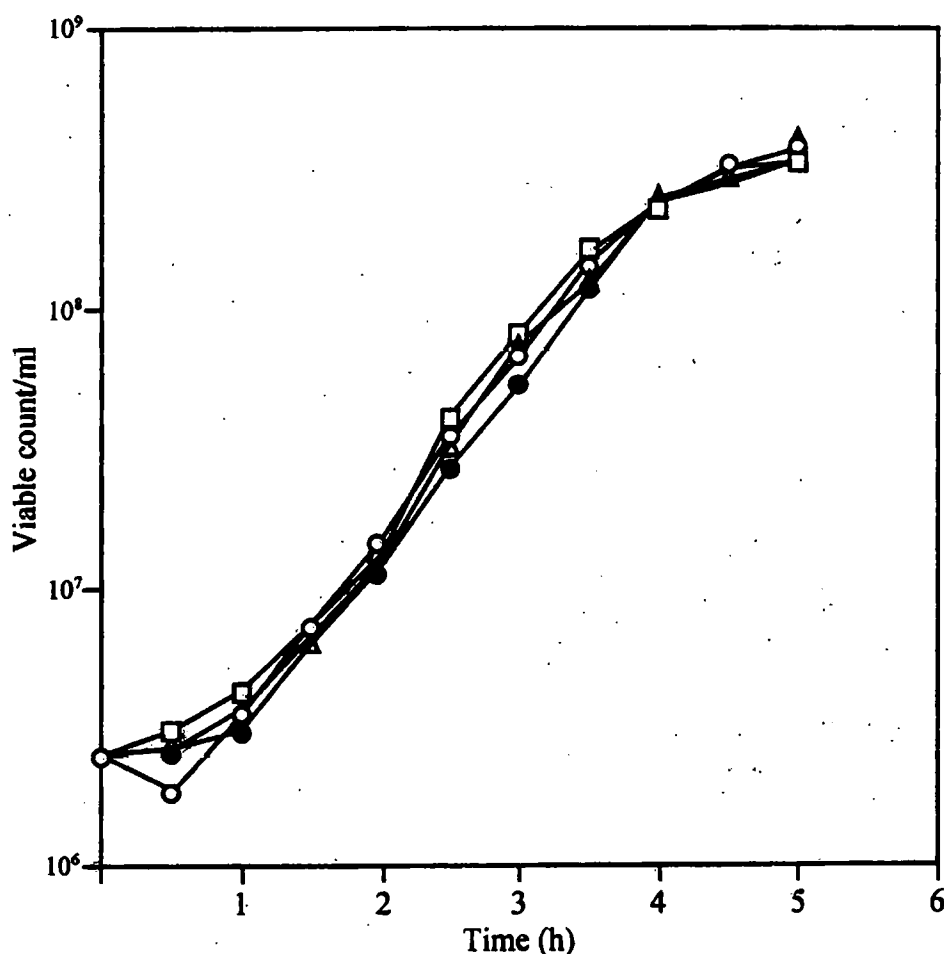
Residual growth of the Sm^D strains CM1242 and CM1253 progenitors of CM1292 and CM1293 under streptomycin deprivation conditions



Growth of the Sm^D strains CM1242 *rpsL832* (○) and CM1253 *rpsL852* (●) (progenitors of strains CM1292 and CM1293 respectively) were followed in L-broth lacking streptomycin. Overnight cultures were spun and washed in phage buffer. Cultures were inoculated with about 2.5×10^3 bacteria per ml and viability was followed for eight hours. Viability was determined by plating appropriate dilutions on L-agar plus streptomycin.

Figure 3.32.3

**Growth of RW202 carrying plasmids pART4, pART6 and pART7
in the absence of IPTG**



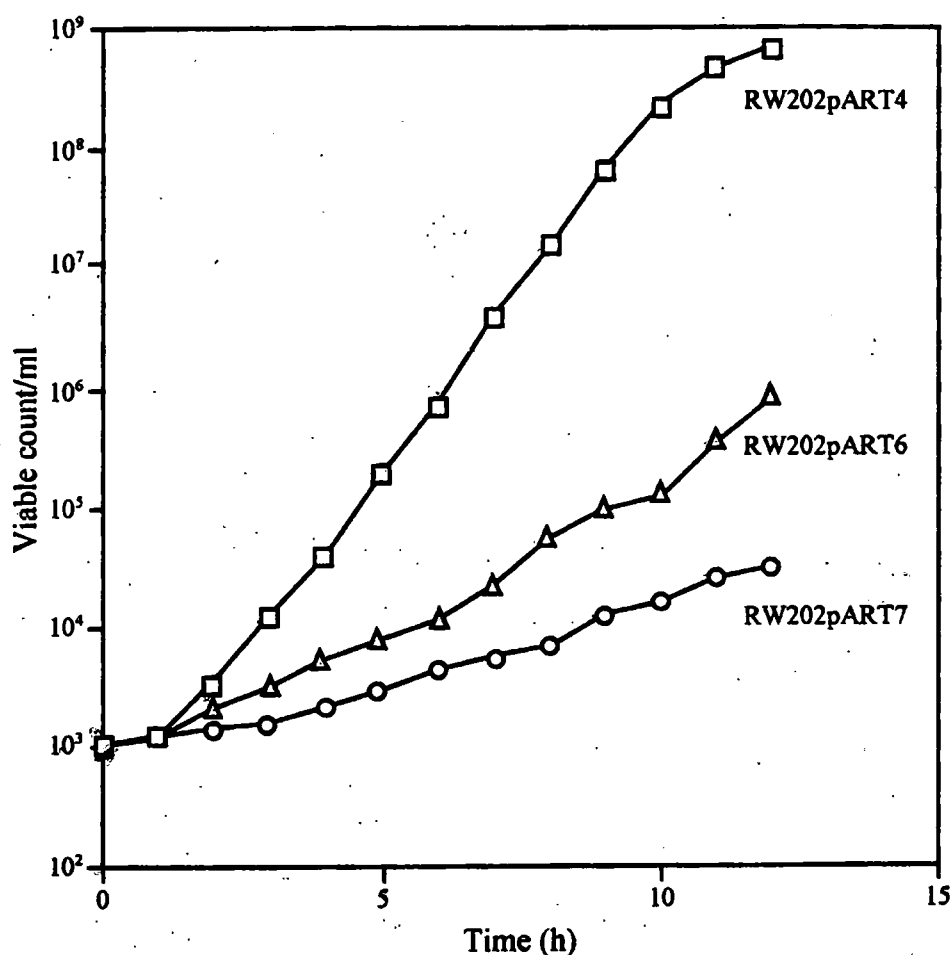
Growth of the strain RW202 (●) alone and with each of the three plasmids pART4 (□, wild type *rpsL*), pART6 (Δ *rpsL852* double mutant) and pART7 (○, *rpsL832* single mutant). Growth was followed in L-broth plus kanamycin, with inocula from stationary overnight cultures. The plasmid borne genes were in the uninduced state *i.e.* the cultures did not contain IPTG. Viability was determined by plating to L-agar plus kanamycin except for RW202 which plates did not contain antibiotic.

The growth of RW202pART4, containing the wild type *rpsL* allele on the plasmid, showed little change on induction, with only a marginal decrease in growth rate and a slightly longer lag phase (35-45 minutes) compared with the uninduced culture (25-30 minutes). However, pART6 and pART7 both conferred a significant reduction in growth rate compared with the induced wild type allele and the equivalent uninduced alleles Figure 3.32.4. Plasmid pART7 containing the single site Sm^D *rpsL*832 allele resulted in the greatest decrease with a doubling time of 1 hour 54 minutes compared to 27 minutes for the induced wild type plasmid. Whereas, plasmid pART6 containing the double mutant *rpsL*852 resulted in an intermediate doubling time of 1 hour 20 minutes. Under these conditions, therefore, the presence of the ancillary mutation would appear to confer a significant selective advantage.

Although Sm^D strains containing only a chromosomal *rpsL*832 or *rpsL*852 allele are not viable in the absence of streptomycin and will not form visible colonies on L-agar plates lacking streptomycin. RW202 containing either of the two plasmids pART6 or pART7 will form colonies on L-agar containing kanamycin and IPTG, albeit slowly. RW202pART6 grows to a colony size of 1-2 mm overnight whereas RW202pART7 requires at least 48 hours to attain a similar size. This growth is ascribed to the continued transcription of the wild type chromosomal *rpsL* gene providing a basal level of functional ribosomes. Even in this situation, *rpsL*852 with the ancillary mutation would appear to be less detrimental to growth than the single site allele.

Figure 3.32.4

**Growth of RW202 carrying plasmids pART4, pART6 and pART7
in the presence of IPTG**



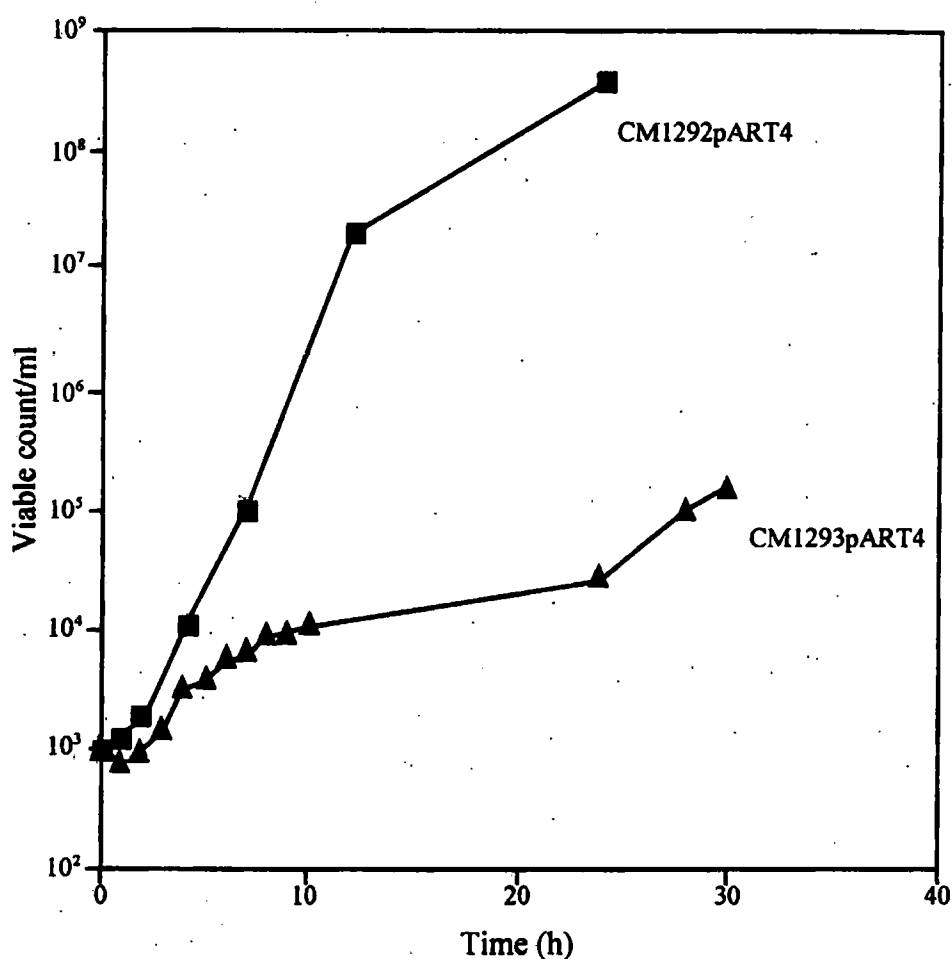
Viable counts of RW202, containing each of the three plasmids pART4 (□, *rpsL*⁺), pART6 (Δ, *rpsL852* double site) and pART7 (○, *rpsL832* single site), upon induction with 1 mM IPTG. Cultures were inoculated with a dilution from a stationary overnight culture in L-broth plus kanamycin; IPTG (1 mM) was added at zero time. Samples were taken at the indicated times and plated to L-agar plus kanamycin. A representative experiment is shown.

When the strain containing induced plasmid pART7 was allowed to continue growing beyond 12 hours the culture was eventually overtaken between 20 and 23 hours after induction by fast growing mutants. These mutants had a growth rate similar to the induced wild type *rpsL* containing strain, the fast growth rate being transferable with the plasmid. Plasmid DNA isolated from a number of fast growing mutants appeared to be the same size to that expected of pART7 and restriction digestion revealed that the *rpsL* fragment remained intact. While a mutation had obviously occurred preventing growth inhibition upon expression of the plasmid borne gene, all that can be said is that it is not due to deletion of the *rpsL* coding region.

The *recA* deletion strains CM1292 (containing *rpsL832*) and CM1293 (containing *rpsL852*) have the same differential in growth rate as that observed for their parents CM1242 and CM1253, an approximate 20-23% growth advantage of the ancillary mutant. However, when pART4 was introduced into CM1292 it had a dramatic inhibitory effect, reducing growth rate by a factor of 4-5. Inhibition occurred in uninduced cultures where expression of Sm^S S12 protein was probably very limited but was still enough to have a pronounced effect. There was no noticeable effect of uninduced pART4 in CM1293 **Figure 3.32.5**. Individual experiments involving CM1292pART4 showed a wide variation in ultimate growth rate, indicating that there was a large selective pressure favouring the growth of cells in which the *rpsL* on pART4 was dysfunctional. The experiment shown in **Figure 3.32.5** presumably represents a colony where *rpsL* on the plasmid is fully functional. Dysfunctional plasmids could be shown to arise in slowly growing cultures of CM1292pART4 at a

Figure 3.32.5

Growth of CM1292 and CM1293 carrying pART4



Growth of the streptomycin-dependent strains CM1292 (\blacktriangle , single site *rpsL832* mutant) and CM1293 (\blacksquare , double site *rpsL852* mutant) containing pART4. Data are from a single representative experiment. Cultures were inoculated with a dilution of an emulsified colony, from fresh transformant plates, into L-broth containing 1.2 mg ml^{-1} streptomycin and kanamycin. No IPTG was added to either strain *i.e.* the wild type *rpsL* on the plasmid was uninduced.

frequency of about 10^{-5} and once arisen rapidly dominated the culture Figure 3.32.6. The CM1292pART4 cultures that exhibited fast growth either contained deletions, assumed to be in the *rpsL* region, or grew rapidly on medium containing streptomycin and IPTG thus indicating non-expression of wild type *rpsL* product. Because of the observed difference between CM1292pART4 and CM1293pART it was found to be unnecessary to induce the plasmid gene with extraneously added IPTG: growth profiles were thus measured with the plasmid gene in the 'uninduced' state. The growth rate of CM1293pART4 was consistent in all experiments performed, with a mean doubling time of 42 minutes. The growth rate was only marginally affected whether the *rpsL* gene on the plasmid was functional as in pART4 or whether it was dysfunctional *i.e.* an inactive mutant (data not shown). CM1292pEH1 and CM1293pEH1 maintained the same growth rate differential as the parental strains without plasmid Figure 3.32.7. This clearly shows that the effect of pART4 on the growth rate of CM1292 is not caused by the maintenance of the plasmid *per se* but must be due to the presence of wild type *rpsL* on the plasmid.

3.33 Discussion

The absence of ancillary mutations at fully redundant codon positions amongst newly arising Sm^{D} mutants argues that the ancillary mutations that have been detected must be presumed to confer some selective advantage, whatever their rate of formation. Potentially there are three areas where such selection could operate in the advantage of the ancillary mutant; i) in a fully expressed Sm^{D} mutant during culture with streptomycin, ii) in a partially expressed Sm^{D} mutant prior to the addition of

**Take over of CM1292pART4 cultures by CM1292 bacteria
containing mutant plasmids**

Growth of the streptomycin-dependent strain CM1292 (▲, single site *rpsL832* mutant) containing pART4. Data is from a single representative experiment. The culture was inoculated with a dilution of an emulsified colony, from fresh transformant plates, into L-broth containing 1.2 mg ml⁻¹ streptomycin and kanamycin. No IPTG was added *i.e.* the wild type *rpsL* on the plasmid was uninduced. The graph is an extension of that shown in **Figure 3.32.5**. Over 99% of the viable cells in the CM1292pART4 culture at 36 hours were found to have a fast growing phenotype when plated to L-agar containing streptomycin, kanamycin and IPTG *i.e.* grew into large colonies overnight. This fast growing phenotype was transferable with the plasmid. In some experiments the plasmid recovered from the culture had obviously undergone a deletion, however, this was not the case in all experiments where, as far as it was possible to determine the plasmid was full sized.

Figure 3.32.6

**Take over of CM1292pART4 cultures by CM1292 bacteria
containing mutant plasmids**

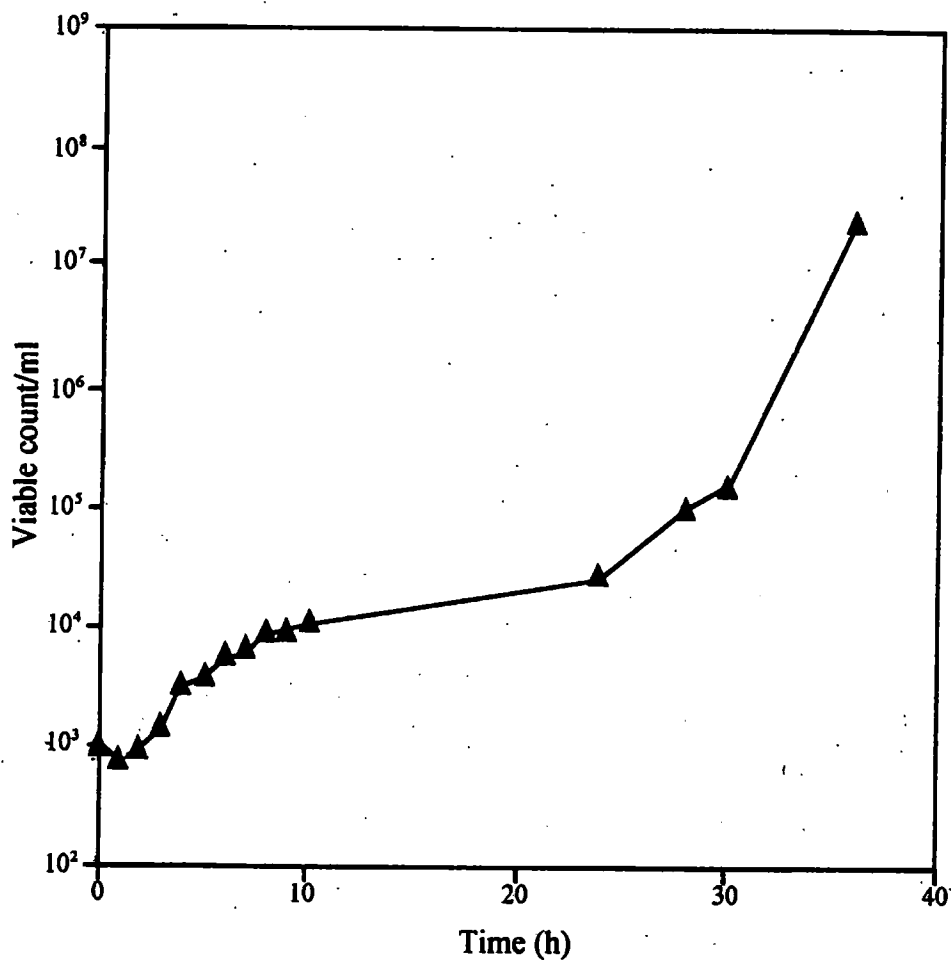
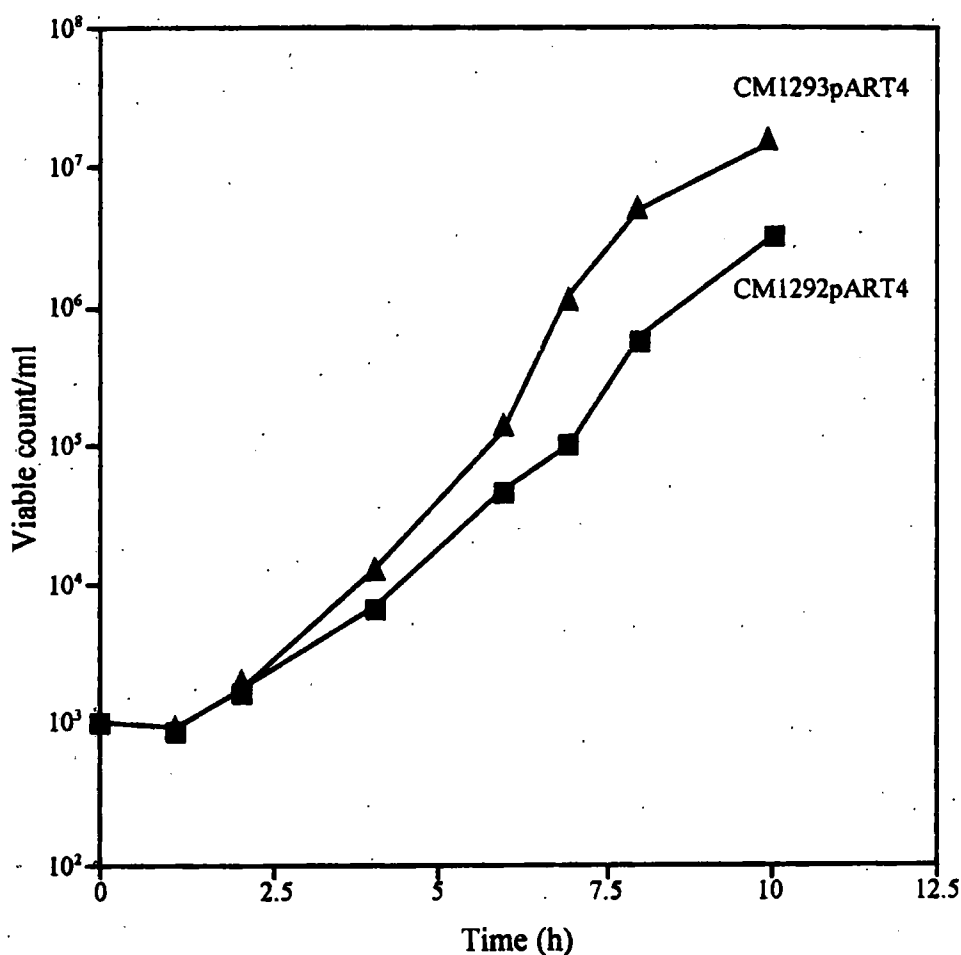


Figure 3.32.7

Growth of CM1292 and CM1293 carrying pEH1



Growth of the streptomycin-dependent strains CM1292 (■, single site *rpsL832* mutant) and CM1293 (▲, double site *rpsL852* mutant) containing plasmid pEH1. Growth data for pEH1 containing strains is the mean of three independent experiments.

streptomycin or iii) in a partially expressed Sm^D mutant after the addition of streptomycin. While some growth advantage attributable to the ancillary mutation has been measured in fully expressed Sm^D strains (Timms and Bridges, 1993)

experiments carried out in the absence of the antibiotic or in cells containing mixed Sm^D and Sm^S ribosomes in the presence of streptomycin has proved to be more complicated.

Established streptomycin dependent strains require streptomycin for growth. While some residual growth is observed when external streptomycin is removed from a liquid culture, this eventually ceases and the viability of the culture declines over time. Here it is interesting that the growth profiles of a single site Sm^D mutant and its complementary ancillary mutant are almost identical when both are deprived of streptomycin, even though the ancillary mutant shows a perceptible growth advantage (~23%) when streptomycin is present (Timms and Bridges, 1993). Thus, there would appear to be some limiting factor common to both strains under streptomycin deprivation conditions that cancels any growth advantage of the ancillary mutant.

Once bacterial ribosomes have encountered streptomycin they tightly bind it such that it cannot be removed by simple washing of the culture (Spotts, 1962). Therefore, residual growth can only occur until the proportion of remaining functional ribosomes, with attached streptomycin, falls to a level no longer sufficient to support continued cell division. Sm^D bacteria deprived of streptomycin can undergo about four to five doublings before growth ceases. This suggests that a cell can continue to divide until over 90% of its ribosomes are non-functional *i.e.* streptomycin free. There is no partition mechanism for ribosomes so, on cell division, some individuals will receive more ribosomes that are functional and consequently will undergo a

greater number of divisions than others. In addition, it is unlikely that washing would remove all surplus streptomycin there could be internal stores available to prolong the period of growth. While a non-dividing cell may remain viable for some further period after division ceases, functional ribosomes are eventually turned over and degraded. Eventually their replacement with streptomycin free non-functional ribosomes reduces the protein synthesis capacity to sub-viable levels.

In the present work, plasmid-based systems were used to mimic the situation in the early stages of the emergence of Sm^D mutant bacteria. The first allowed observation of the effect of expression of Sm^D alleles in the absence of streptomycin while; the second allowed expression of Sm^S ribosomes in a Sm^D background. Both have relevance to the situation found in formation of spontaneous Sm^D mutants where individual cells contain a mixture of resistant and sensitive ribosomes which affect behaviour both before and immediately after the addition of streptomycin.

When a doubly mutant *rpsL* gene on a plasmid is expressed in the presence of a chromosomal *rpsL*⁺ gene, bacteria can grow faster than when the plasmid borne gene is singly mutant. In the context of a newly emergent Sm^D mutant, growing in the absence of streptomycin, this would imply a selective advantage for bacteria carrying an ancillary mutation in *rpsL*. This advantage is necessarily limited in duration and would only apply during the period before Sm^D ribosomes accumulate to a level at which growth would cease anyway.

Assuming there are a finite number of divisions that can occur before a Sm^D mutant stops growing the mutation rate would have to be very high, of the order of 10^{-1} to 10^{-2} . This figure is obtained by extrapolating data from Sm^D strains that are allowed to continue growing in media where streptomycin has been removed. Under these conditions, established Sm^D strains undergo between five and six doublings before growth ceases, presumably due to dilution of functional ribosomes retaining bound streptomycin. If this situation were analogous to the accumulation of Sm^D ribosomes in newly expressed Sm^D mutants in the absence of streptomycin, residual growth would depend on the relative proportions of Sm^D and wild type ribosomes in the cell perhaps allowing five to six doublings before growth ceased. In this case, the largest clone size of Sm^D mutants that could accumulate in a culture lacking streptomycin would be 64 (2^6). Once cultures are plated out on streptomycin-containing agar, results from our second model indicate that growth of singly mutant Sm^D bacteria would rapidly cease in the presence of wild type ribosomes. However, those with ancillary mutations would continue to grow albeit at a slower rate. Eventually both types of mutant would dilute the wild type ribosomes to a level at which growth could resume and the mutants form visible colonies

The selective advantage conferred by the ancillary mutation, in the presence of streptomycin, is necessarily limited to the period before ribosomes containing wild type S12 are diluted by cell division or degraded. Moreover, to experience such extreme selective advantage the ancillary mutation would have to arise during the early part of this period, soon after the newly expressed Sm^D mutant was plated onto streptomycin. Cells with ancillary *rpsL* mutations have frequently been observed to

occur in mixed colonies, with cells carrying the same Sm^D mutation but lacking the ancillary mutation. This has also been found to be true of the two alleles studied in this section. It is presumed that in a mixed colony, the Sm^D mutation in *rpsL852* must already have been present at the time of plating on streptomycin agar, and the ancillary mutation must have arisen subsequently. The selective advantage conferred by the ancillary mutation in *rpsL852*, when established strains are growing in streptomycin medium, was ~23% (Timms and Bridges, 1993). It was estimated that, with a 23% growth rate advantage, the ancillary mutation must have arisen when the colony size was between 10^3 and 10^4 bacteria. This implies a mutation rate for this locus of the order of 10^{-3} to 10^{-4} /replication (Timms and Bridges, 1993).

If ancillary mutations arose with a frequency as high as $\leq 10^{-1}$ to 10^{-2} , they probably experience the selective advantage, documented in this work, when wild type ribosomal S12 protein is present along with Sm^D ribosomes. Together with the previously reported selective advantage for established strains, the expected outcome would be that bacteria with ancillary mutations would outgrow singly mutant Sm^D bacteria and would dominate the streptomycin dependent colony. Domination by the ancillary allele would also be the likely outcome if the ancillary mutation rate were of the order of 10^{-2} to 10^{-3} /replication. In this case the selective advantage associated with the presence of the wild type ribosomal protein would be very much reduced, as the Sm^S ribosomes would have been diluted by ~7 to 10 bacterial divisions. As previously calculated, ancillary mutation rates of 10^{-3} to 10^{-4} would be likely to result in colonies containing comparable numbers of singly and double mutant bacteria. Therefore, since approximately 20% of newly arisen mutant colonies are detectably

mixed, it is assumed that the ancillary mutation rate is likely to be nearer to 10^{-4} than 10^{-1} . Mutation rates of $\geq 10^{-4}$ /base pair are, of course, far too high to occur over the whole genome for extended periods, the result would be inviability. However, such a rate of mutagenesis could be survived providing either i) it were only transiently expressed or, ii) it was only effective over a localised area of the chromosome.

One of the predictions of the transient model is that there are factors operating in newly arisen Sm^D mutants that increase the mutation rate in *rpsL*. However, once the phenotype is fully expressed, the mutation rate is presumed to revert to a more typical $\sim 10^{-10}$ /base pair/replication. What factors exist in a newly expressed Sm^D cell that could cause a transient increase in mutation rate? Streptomycin is known to adversely effect translational accuracy, both *in-vitro* and *in-vivo* (Davies *et al.*, 1964, Boe, 1992), by interfering with the proof-reading function of wild-type ribosomes (Ruusala and Kurland, 1984). The resulting misreading of the genetic code can lead to streptomycin-conditional suppression or phenotypic masking of some auxotrophies (Davies *et al.* 1964, Gorini and Kataja, 1964a; 1964b; Gorini, 1971; Edelmann and Gallant, 1977; Bouadloun *et al.*, 1983; Johnson and Parker, 1985). These effects are only seen when streptomycin is added to wild type ribosomes most Sm^D and Sm^R ribosomes display increased or hyper-accurate translational fidelity (Bohman *et al.*, 1984; Ruusala *et al.*, 1984; Andersson *et al.*, 1986).

In newly expressed Sm^D mutants (and in Sm^R mutants formed during the last few generations before a culture goes stationary), there will still be a proportion of wild type ribosomes in the cell. Boe (1992) found that wild type cells treated with a low

level 5µg/ml of streptomycin showed a 10^6 fold drop in viability, however, of the survivors 0.1% showed measurable auxotrophies. Mistranslation by these streptomycin-sensitive ribosomes in the presence of streptomycin could result in the formation of a mutator protein; for example, a DNA polymerase enzyme with reduced proof-reading capabilities. Since the mutator activity would not be genetically heritable, the effects would only be transiently expressed until the aberrant protein was degraded. Removal of the epsilon proof-reading subunit from DNA polymerase III typically results in an increase in mutation rate of between 10^2 to $>10^4$ fold when *E.coli mutD5* or *dnaQ49* mutants are grown in rich medium at 37°C (Fowler *et al.*, 1974; Horiuchi *et al.*, 1978; Fowler *et al.*, 1986; Krishnaswamy *et al.*, 1993). Although high, a mutation rate of this magnitude is still somewhat short of the 10^5 to 10^6 fold increase in rate that is observed in *rpsL*.

An alternative is that the wild type ribosomes 'poisoned' by streptomycin could have a direct effect on the level of mutagenesis within a cell. Transcribing RNA polymerases have been linked to transient arrest of replication forks (Liu and Alberts, 1995; Deshpande and Newlon, 1996) and transcribed regions are prone to enhanced deletogenesis (Vilette *et al.*, 1992; 1995; 1996). Perhaps poisoned ribosomes could interfere with the transcriptional apparatus of the cell leading to blockage of an advancing replisome and collapse of the replication fork. Although collapse of DNA replication forks has been linked to increased mutagenesis (Bierne *et al.*, 1991; Bierne *et al.*, 1997a; Michel *et al.*, 1997; Seigneur *et al.*, 1997) the mutagenic events described are mostly deletions rather than the point mutations seen in *rpsL*. However, because the processes of transcription and translation are so closely linked in bacteria

such interactions are feasible and a stalled transcriptional/translational complex would be a substantial obstacle to an advancing polymerase or to post replicational repair mechanisms. There is some evidence that ribosomes and RNA polymerase do directly effect the activity of each other, perhaps indicating a physical interaction between both complexes (Chakrabarti and Gorini, 1975; Chakrabarti and Gorini, 1977; Schmitt *et al.*, 1995). In addition, the number of poisoned wild type ribosomes could potentially be very large, providing plenty of scope for multiple interactions and increasing the opportunity of mutagenic events occurring.

Both of the potential processes outlined above could result in a general increase in mutation rate throughout the chromosome but would be limited in duration until the wild type ribosomes had been diluted from the system. At a mutation rate of 10^4 to 10^6 /base pair/generation non-selected mutations should be detectable in newly arising Sm^D or Sm^R mutants. There are perhaps 100 genes in which a forward mutation will lead to a detectable auxotrophy. Screening of sufficient numbers of Sm^D and Sm^R mutants would quickly establish whether the apparent increase in mutations rate in *rpsL* was also occurring elsewhere in the chromosome. Mapping and analysis of any auxotrophs recovered could yield important information on the mutational process. Questions that could be asked include; is mutagenesis confined to transcribed genes? Does the process show any spatial relationship with respect to *rpsL*? What rate are auxotrophies recovered, which could provide independent verification of the mutagenic rate calculated for *rpsL*.

The second option is that the increased mutagenesis observed is confined to the *rpsL* region alone. There exist examples of preferentially localised mutagenesis *e.g.* in the *recA* gene (Liu and Tessman, 1990; Liu *et al.*, 1993), in *mutS* containing a *lacZ* insertion (Liu *et al.*, 1997) and during starvation of *E.coli* for asparagine resulting in reversion of *asnA* and associated mutations at a linked locus *atp* (Boe, 1990). In the case of *recA* a specific allele, *recA1202*, exhibits a general mutator phenotype elevating chromosomal mutation rates by about 100 fold however, the mutation frequencies within the *recA* gene itself are increased by 10^4 to 10^5 fold. In contrast, the specific *mutS* insertion isolated by Liu *et al.* (1997) exhibits very highly localised increased mutation frequencies with little increase in concomitant general mutagenesis. Both gene products have DNA binding capabilities; one hypothesis for their localised effect is that they form aggregations and bind near to their sites of production (translation) such aggregations may interfere with normal DNA repair functions or DNA replication. Could *rpsL* also agglutinate near to its own site of production? The Sm^D mutations recovered undoubtedly confer a conformational change on the relatively small S12 protein. If the new conformation were to be 'sticky' as soon as S12 was produced, it could aggregate to form particles that may then interfere with repair or replication processes. Presumably once produced, S12 protein would be incorporated into ribosomes fairly close to the site of their production. Once in ribosomes the stick effects would be ameliorated localising the effects on replication or other mutagenic processes. If this proved to be the case, an interesting question is; do the ancillary mutations merely confer a selective advantage or do they also reduce 'stickiness'? If the latter then the effect could be

described as localised and transient, in that as soon as a mutation occurred mutation rates would return to normal.

What evidence is there that the *rpsL* region is subject to a localised increase in mutation rate? If mutations were occurring randomly in *rpsL* at the same time that the ancillary mutations arise, at least some of them should persist in particular at fully redundant codon positions (roughly one quarter of the total). Most mutations would result in an amino acid change and are likely to be deleterious since the Sm^{D} phenotype can only express itself in a functional protein; these mutations would not be seen. In a situation where the mutation rate for ancillary mutations is 10^{-4} (the lowest of the above estimates), the probability of a mutation occurring at a fully redundant codon position elsewhere in the gene should be $\sim 0.25 \times 10^{-4}$ /base pair. 150 base pairs were routinely screened, so the probability of seeing such a mutation accompanying an ancillary mutation would be $0.25 \times 10^{-4} \times 150 = 3.75 \times 10^{-3}$. 32 Sm^{D} mutants with ancillary mutations have been screened, mostly using single stranded-conformational polymorphism analysis. Assuming a detection sensitivity for a single base change of at least 0.9 then 0.1 ($3.75 \times 10^{-3} \times 32 \times 0.9$) additional mutations at a fully redundant codon position might have been detected compared to the zero observed. Clearly many more mutants (base pairs) would have to be examined to assert with any confidence that such mutations are absent.

Whether random or not, the rate of occurrence of ancillary mutations in *rpsL* is many orders of magnitude higher than the rate expected ($\sim 10^{-10}$ /base pair/replication) if they were arising totally independently of the accompanying Sm^{D} mutations. These

mutations are thus temporally clustered, indicating that the *rpsL* region (at least) experiences periodic episodes of hypermutation. Many questions remain to be answered about this phenomenon. Perhaps the most important is whether clustering is a general feature of spontaneous mutation or reflects a hypermutable stress state triggered by the expression of the first mutation, in this case streptomycin dependence.

4 General Discussion

The results presented in this thesis show several ways that bacteria respond to the imposition of selective stress. The types of mutations that arise under conditions of stress are different from those arising under non-selective conditions as indicated by the small in-frame deletions arising in *trpA* under tryptophan starvation. They also imply that the causes of spontaneous mutations under starvation conditions are different. Most mutations in growing bacteria are assumed to result from polymerase errors that escape repair by MMR or from repair synthesis following DNA damage. The results with the *trpA* system suggest that oxidative damage is very important in non-growing cells, perhaps because repair processes are compromised due to the low rate of protein synthesis. Inducible repair responses such as SOS are also likely to be compromised for the same reason although induction of SOS has been shown to occur in old colonies.

While the results do not provide direct evidence, there is indication that DNA secondary structure is very important in the formation of some types of mutations

especially deletions. *E.coli* does not tolerate long palindromes, which are very unstable in SbcDC proficient strains. The effect of pseudo-palindromes on viability and mutagenesis is less certain. However, all the deletions observed in *trpA* occur in the vicinity of two potential regions of secondary structure. In stationary cells where the frequency of replication and presumably of transcription is reduced, DNA may be more prone to formation of structures that can then provide the targets for deletogenic mechanisms. It would be interesting to assess the contribution of such structures to stationary phase mutagenesis and finding the proteins or pathways responsible for producing deletions. An attempt has been made to adapt a plasmid based deletion system for use in starving cells but with little success. The particular system involved mutation to antibiotic resistance by deletion of one repeat from a two direct repeat structure. In practice, it was found that expression of antibiotic resistance was necessary and that there was too much residual growth during this expression period. It was thus impossible to differentiate mutations resulting from residual growth from those induced during the starvation period.

The persistence of mismatched base pairs has also been shown to increase the yield of deletions recovered but again whether it is the presence of the mismatches that is important or the results of some aberrant repair process is uncertain. Perhaps mismatches serve to stall replication forks, a process that may be mutagenic and lead preferentially to deletions, possibly *via* means of a double strand break. Under conditions of starvation, repair of such breaks may not proceed with such efficiency as in growing cells. The formation of deletions in *trpA* did not appear to require functional RecA, but the effects of other recombination proteins were not tested in

particular RecB, RecC or RecD which are important in double strand break repair. Alternatively, the lack of mismatch repair may increase the level of illegitimate recombination between homeologous sequences. Again, whether such recombination is stimulated by secondary structure, single stranded DNA produced by repair processes, repair of double strand breaks, or slippage during replication is unclear.

Several studies have shown that tyrosine independent mutations arise when the *tyrA14* strain WU3610 is incubated for upwards of six days on minimal agar plates. Display of this phenomenon has been shown to be dependent on the presence of the gene *tas*, which complements the prephenate dehydrogenase activity lacking in WU3610. The studies have not shown whether *tas* is the target gene, although a direct mutation has been ruled out by sequencing. Regulation of *tas* gene expression may thus be responsible for the observed phenotype. Mutator activity, in a *mutL* strain, increases the yield of slow growing mutants suggesting that a mutation is involved in the process. As mutations at the *tas* locus has not been demonstrated a mutation in another gene would seem to be responsible. There are a number of genes that could affect *tas* expression by a variety of different mechanisms. These genes could be sequenced directly or, if gene inactivation is responsible, an insertion library could be constructed providing an effective selection was available for screening.

Are there comparisons to be made between the tryptophan and tyrosine starvation systems? Both indicate the variety of responses that bacteria can muster to overcome challenges in the environment. The deletion system may highlight an underlying

mechanism that could be harmful to bacteria in most cases. After all, it is more difficult to reacquire deleted sequences than duplicate or mutate them. In this defined situation, deletions are advantageous but if they occurred elsewhere, deletions may be a major source of inviability in starving bacteria. Compensation of tyrosine auxotrophy by a gene that presumably is not normally involved in tyrosine biosynthesis displays an interesting redundancy in *E.coli*. Is this a case of good fortune or does the inherent non-specificity of many biological reactions indicate that in some respects certain enzymes can be jack of all trades, limited only by competition for substrates?

As individuals *E.coli* are expendable and sacrifice of individuals in search of an adaptation may allow the species to expand and exploit available resources. This illuminates the question at the heart of adaptive mutagenesis, which is whether *E.coli* (and other bacteria) have a means to enhance mutagenesis under conditions of severe selection, perhaps this is dependent on one of the global regulators of bacterial physiology. Cell physiology is likely to be very different in starving bacteria and may affect the fidelity of replication, transcription and translation, all processes that may alter the rate at which bacteria mutate. There is some evidence that a proportion of cells under stress can enter a period of hypermutability, increasing the mutation rate throughout the genome. The result of this is that occasionally mutations are produced in a sub-population that can utilise resources unavailable to the general population.

The remaining section of the thesis examines the mutations arising in streptomycin

dependent bacteria. A high proportion of newly arising Sm^D bacteria shows ancillary mutations accompanying the Sm^D determining mutation. These are recovered at a rate far higher than can be accounted for by selective advantage of the ancillary mutant compared to the primary Sm^D mutant. This appears to be a directed process in that a general increase in mutation rate has not been demonstrated in newly arising Sm^D bacteria, nor has elevated mutation rates been demonstrated in established Sm^D strains.

Of the three systems discussed, this one has proved the most refractory to easy exploration. The techniques of single stranded conformational polymorphism (SSCP) and sequencing, used in Timms *et al.* (1992) and Timms and Bridges (1993), are time and labour consuming and have the disadvantage that they can only screen relatively small DNA fragments. They are therefore unsuited to answering some of the questions that are presently outstanding. For example, if *rpsL* in newly arisen Sm^D bacteria shows hyper-mutation, does this extend to other parts of the chromosome or is it localised to *rpsL* and its immediate surrounds. One way that this question may be answered is to screen a number of new Sm^D mutants for auxotrophies, it would then be relatively easy to map any mutations found. This would provide information on the distribution of mutations and possibly on the types of mutation occurring. If mutagenesis was found to be localised a modified form of SSCP, looking at larger fragments of DNA, could examine any spatial distribution of mutations around the *rpsL* gene without the need for a selectable mutant phenotype. *In vitro* transcription/translation systems could also be used to examine the possibility that Sm^D S12 protein is "sticky" and agglutinates near to the site of its

production. Some form of gel retardation assay would indicate whether this was the case. Perhaps such a system could be modified to look at possible interactions between ribosomes and RNA polymerase, and the effects of Sm^D and ancillary mutations on such potential interactions.

Are these three cases related? Is the apparent hypermutation of the *rpsL* locus achieved by the same mechanisms that produce starvation-associated mutations in the amino acid starvation systems? One can attempt to construct a model to explain some aspects of the phenomena observed based on recent work. Stationary phase mutation in the episomal *lacZ* system has been linked to the repair of double strand DNA breaks (Rosenberg *et al.* (1996). Could these also be involved in the starvation-associated systems?

Evidence is available to suggest that replication forks collapse when they encounter some forms of DNA damage or secondary structure, which causes pausing or slowing of the replication complex (Kuzminov, 1995a). Michel *et al.* (1997) have shown that such events can lead to double-strand breaks at the stalled replication complex. The repair of double-strand breaks has been linked to increased mutagenesis at the site of breakage. One model to explain hyper-mutability at *rpsL* also invokes DNA replication fork arrest, involving a stalled ribosome/RNA polymerase complex or agglutinated Sm^D *rpsL* protein. In starving cells, the probability is that restrictions in the supply of precursors may cause frequent pausing of the replication fork and lead to its collapse. It may be significant that *dnaN* and

recF, two genes required for the recovery of stalled replication forks, are induced on entry into stationary phase (Villarroya *et al.* 1998).

Several starvation-associated mutation systems including reversion of amino acid auxotrophies seem to require some leakiness for the appearance of mutants. This leakiness may be necessary to provide energy and precursors for DNA synthesis as well as expression of the mutant phenotype. Bridges (1996a) has presented evidence that suggests that there is more DNA synthesis occurring in starving cells than has previously been recognised, presumably this synthesis is prone to frequent replication fork collapse. This raises the question, what form does this DNA synthesis take? Is it for example initiated from *oriC*, the chromosomal origin of replication? If this is the case, one could expect to see a spatial distribution of mutations around the origin, with more mutations occurring closer to *oriC*. However, if the synthesis was some form of stable DNA replication *i.e.* not dependent on initiation at *oriC*, the mutants could be spread randomly around the chromosome. Thus, there are still many questions that need to be answered before links between the systems can be unambiguously identified.

5.1 Appendix I: The *tyrT* locus of *Escherichia coli* B/r

PCR and Sequencing of *tyrT*

Culture conditions and boiling steps were as previously described. PCR was performed as previously described for 30 cycles with 100 pmoles of the following primers; TT256 (5'XGTTATTCATGTCGATCATACCTACAC3') and primer TT1625 (5'XTTTTAGTTTCACATAGACCCTGCTTC3'). Both primers were synthesised with and without 5' biotinylation (X) to allow single strand sequencing of both DNA strands. Dynabead aided strand separation and sequencing were as previously described. Sequencing primers were initially designed using the *E.coli* K-12 sequence and supplemented with sequence information produced from strand walking.

It has been reported that tyrosine auxotrophy, in the *tyrA14 E.coli* B/r strain WU3610, can be suppressed by an unknown gene (Bridges, 1994). The slowly growing suppressor containing mutants were detected under conditions of tyrosine starvation but they were also shown to arise in growing bacteria if appropriate experimental conditions were applied. Most tyrosine independent mutants arising under growth conditions result from base pair substitutions at the ochre site (Li *et al.*, 1991) or at tRNA suppressor loci (Osborne and Person, 1967) and have a fast-growing phenotype. Because the majority of the tyrosine independent mutants arising during starvation were slow-growing most of these suppressor or revertant mutants could be ruled out (Bridges, 1994) and the base-pair substitution options were

narrowed to transversion at a G:C base pair. A candidate locus susceptible to such a change is *tyrT*, coding for tRNA₁^{Tyr} and requiring a G:C→T:A base change to produce an ochre suppressor (GTA→TTA). In *E.coli* K-12 strains, mutations at the *tyrT* locus are known to give rise to both amber and ochre nonsense suppressors (Signer *et al.*, 1965; Goodman *et al.*, 1968). However, during their study of suppressor and back mutations of the *tyrA* locus of WU3610, Li *et al.* (1991) did not find any ochre suppressors that mapped to *tyrT*. It was therefore thought that mutation at this locus could be a candidate for producing the slow-growing Tyr⁺ mutants observed in WU3610. The *tyrT* sequence was determined for six slow growing Tyr⁺ mutants, isolated at day nine from a starvation experiment, all were found to be wild type, *i.e.* they did not contain suppressor mutations in either of the *tyrT* α or β tRNA₁^{Tyr} copies.

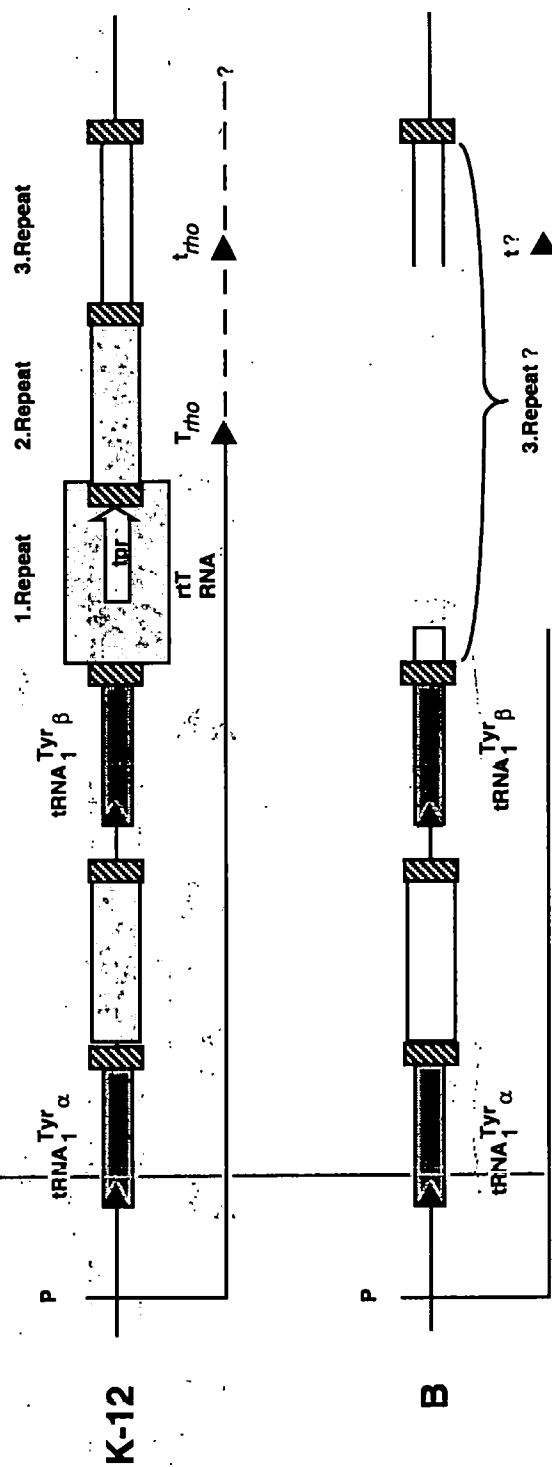
PCR primers were designed, using the previously sequenced *E.coli* K-12 region (Accession number K01197), to give an expected fragment size of 1,393 base pairs. It was noticed that when the product from the B/r strain was run on an agarose gel it appeared to be 350-400 base pairs smaller than expected, the K-12 PCR product was of the anticipated size. This deletion, of 357 base pairs, was confirmed when the product was sequenced and subsequently the entire *tyrT* fragment from the B/r strain WU3610 was sequenced (Accession number X90989). The structure of the *E.coli* K-12 *tyrT* region is shown in Figure 5.1.1 along with the equivalent region for WU3610 as determined by sequencing. This region is known to be unstable when carried on the transducing phage ϕ 80 (Russell *et al.*, 1970; Egan and Landy, 1978; McCorkle and Altman, 1982).

Structure of the *tyrT* locus from *Escherichia coli* K-12 and B

The K-12 diagram was adapted from the work of Bösl and Kersten (1991), and the B diagram follows their conventions. The promoter (P), the *rho* termination sites T_{rho} (major) and t_{rho} (minor), the two $tRNA_{I}^{TYR}$ genes (darkly shaded boxes), the terminal repeats, and *rT* RNA (grey boxes) are indicated in the *E.coli* K-12 diagram. The dotted line that continues beyond the t_{rho} terminator indicates the observation that some transcripts are terminated beyond any of the recognised termination signals. The hatched boxes indicate a 19 base pair motif (AATCCTTCCCCCACCACCA) that occurs a total of six times in the K-12 sequence, including the terminal 19 base pairs of each $tRNA_{I}^{TYR}$ coding sequence. The polypeptide Tpr is indicated as an arrow in the first repeat. The lower diagram indicates the structure of the *E.coli* B locus, showing the absence of repeats 1 and 2. While the possibility that the B sequence actually derives from either repeat 1 or 2 or portions thereof cannot be ruled out, the total sequence obtained showed more overall homology to the third repeat of K-12 than to the other two.

Figure 5.1.1

Structure of the *tyrT* locus from *Escherichia coli* K-12 and B



Therefore, the possibility that the strain WU3610 had undergone a deletion at *tyrT*, during its derivation, and that the structure of this locus in other members of the B family corresponds to that in *E.coli* K-12 was considered. In our laboratory stocks, there were two *E.coli* B strains that were acquired around 1957 (B_{ALPER}) and 1969 (B_{CSH}) and have not subsequently been manipulated in any way apart from propagation into new stab cultures. PCR was performed on both strains and both proved to have the same sized product as WU3610. It is likely therefore that this deletion is widely present in the B family.

Comparison of the K-12 and B/r sequences indicated that there were 10 base pair substitutions in the 987 bases sequenced; eight transitions and two transversions, as well as one base pair deletion and one base pair insertion. The base pair deletion and insertion both occurred in the intervening sequence between the two tRNA genes maintaining the net 208 base pair spacing. The sequences of the two tRNA genes were identical between K-12 and B/r strains. The major difference, however, was the absence in the *E.coli* B/r locus of two of the 3.14 terminal repeat sequences. Genomic DNA from WU3610 was probed using the full length PCR product from an *E.coli* K-12 strain. The blot confirmed the structure of the *tyrT* region in the B family and showed that the deleted sequences have not been translocated elsewhere in the chromosome.

Termination of *tyrT* transcription is ρ (Rho) dependent in *E.coli* K-12. The sequence, corresponding to the major and minor ρ termination sites, (5'CAATCAA3') is present in the second and third repeats of the *E.coli* K-12 *tyrT* locus (Küpper *et al.*, 1978;

Rossi *et al.*, 1981). However, the equivalent sequence in the *E.coli* B/r locus (5'TAATTAA3') contains two base changes such that it bears a closer resemblance to the sequence found in the first repeat of *E.coli* K-12 (5'CAATTAA3'). It is uncertain whether this site functions as a minor termination signal in *E.coli* K-12 or whether it is a ribonuclease-processing site involved in maturation of the tRNA (Rossi *et al.*, 1981). Thus, it is possible that these sequences are not involved in transcription termination and that termination of the *tyrT* transcript in *E.coli* B/r occurs at a downstream site. Alternatively the sequence in *E.coli* B/r may be a ρ dependent termination signal but this would only become apparent were the termini of the *tyrT* transcript in *E.coli* B/r determined experimentally. The 33 amino acid basic polypeptide (Tpr), coded for within the first terminal repeat in *E.coli* K-12 (Altman *et al.*, 1981; Rossi *et al.*, 1981), would also appear to be absent from *E.coli* B/r as a termination codon is present 10 residues into the equivalent coding sequence. When the sequences of the terminal repeats in *E.coli* K-12 and those found in B/r were compared, the *E.coli* B/r sequence appeared to bear more overall homology to the third repeat in K-12 than to either of the other two repeat sequences. The possibility exists that the *E.coli* K-12 *tyrT* locus is the ancestral structure while *E.coli* B/r *tyrT* derived from it by a process of unequal recombination, resulting in deletion, after the two strains had diverged. If this is so, the remaining terminal repeat in *E.coli* B/r may be derived from portions of more than one repeat. Alternatively, amplification of the repeats may have occurred in *E.coli* K-12 from an ancestral locus similar to that in *E.coli* B/r.

It has been stated that in previous studies, in *E.coli* B/r, no ochre suppressors have

been mapped to the *tyrT* locus. While the present work was in progress Urios and Blanco (1996) reported the isolation of ochre suppressors at this site in experiments with strains deficient in *mutY*. These isolates were able to suppress ochre nonsense mutations in the T4 bacteriophage oc427 and suppression was co-transducible with a marker linked to *tyrT*. One of their isolates (IC5015) was obtained and sequenced as above. It confirmed that the sequence of the *tyrT* tRNA α and β coding regions were identical to that obtained for WU3610, apart from a G:C→T:A change in the anticodon of the *tyrT* β copy. There are two points worth noting about IC5015: i) it carries a deficiency in *mutY* which predisposes it to accumulate G:C→T:A transversions (Moriya and Grollman, 1993; Bridges *et al.*, 1996) which is the base change required for ochre mutations at *tyrT* and ii) the strain is a K-12 / B hybrid. While its *tyrT* locus is definitely B in origin at least six exoribonucleases, which may have different sequence or structural specificity in K-12 and B strains, are known to be involved in tRNA maturation in *E.coli* (Li and Deutscher, 1996). It is possible that during hybrid formation K-12 genes specifying one or more of these enzymes may have been integrated into the *E.coli* B genome. If they were to affect the processing of the *tyrT* precursor, they could allow expression of a suppressor phenotype. In this context, it would be interesting to see whether the ochre suppressor remained functional when it was transferred into a pure *E.coli* B genetic background.

Further examination of the results of Urios and Blanco (1996) reveal that in a *mut*⁺ strain, in the absence of *umuDC* or functional homologues, there were no spontaneously occurring ochre suppressors at *tyrT*. These results suggest that, unless the mutagenic processes are heavily weighted in their favour *e.g.* inactivation of

mutY or overexpression of mutagenic repair proteins, mutations producing ochre suppressors at *tyrT* are rare in the *E.coli* B background. A further factor may be that Urios and Blanco (1996) looked at mutations suppressing the *trpE65* allele in their system, not the *tyrA14* allele examined by Li *et al.* (1991) and in this thesis. A simple explanation for the non-observance of *tyrT* ochre suppressors, in the *tyrA14* system, is that insertion of tyrosine at the site of the ochre mutation in *tyrA* does not produce a functional protein.

If the terminal repeat sequences provide crucial stability to the nascent transcripts, one can speculate that the structure of the *tyrT* locus in *E.coli* B may affect stability of the tRNA precursor. In K-12 strains, both gene copies are processed to mature tRNA allowing a suppressor mutation in one copy while maintaining wild-type recognition in the other while, in *E.coli* B strains the locus may be functionally monocistronic. The transcript may be degraded before processing is complete, requiring the presence of two wild-type tRNA genes for viability, or the mutations noted may affect the processing sites required for generation of mature tRNA from one or both tRNA genes. In this scenario the second tRNA gene is more important in providing stability to the transcript and allows expression of sufficient tRNA_I^{TYR} for viability. Alternatively, the absence of suppressors may be an indirect consequence of the lack, in B strains, of *rtT* RNA or the basic polypeptide Tpr. Both of these appear to be involved in modulation of the cellular response to amino-acid starvation (Michelsen *et al.*, 1989; Bösl and Kersten, 1991) and which may affect the expression of suppressors at the *tyrT* locus under certain conditions.

5.2 Appendix II: A presumptive *rpoS* mutant allele in an isolate of the *E.coli* B/r strain WU3610

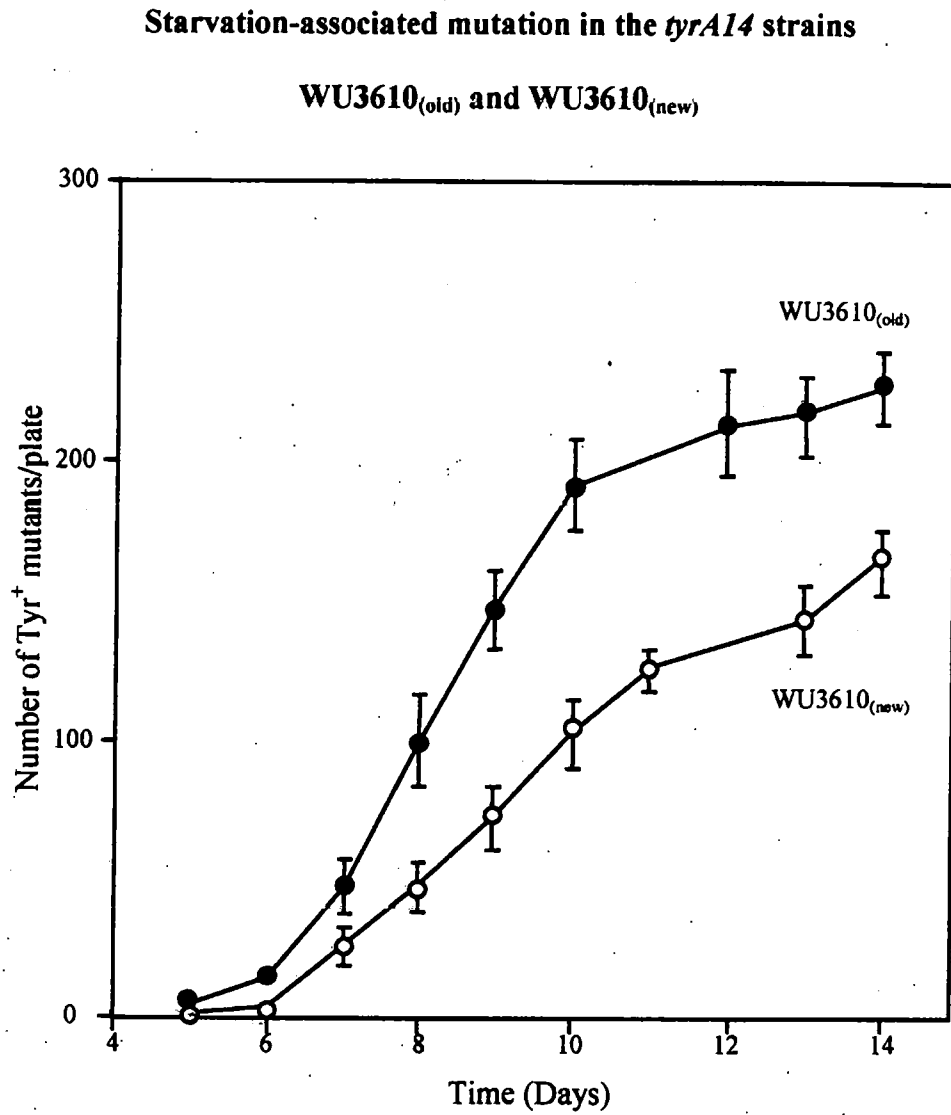
One of the strains used in this thesis is the *E.coli* B strain WU3610, which is known to contain two amino acid auxotrophies conferred by the *leu308* and *tyrA14* alleles. However, during an investigation into starvation-associated mutagenesis in this strain it was suspected that there might be a further mutation present. When the *rpoS::Tn10* allele was transduced into recipient strains it was standard practice to confirm the transductant contained the required allele by using a simple phenotypic test. This was a comparison of the rate at which bubbling occurred on ten day old colonies grown on L-agar plates after addition of a drop of 30% hydrogen peroxide (H₂O₂).

E.coli contains two hydrogen peroxidases HP I and HP II coded for by the genes *katG* and *katE* respectively. HP I is constitutively expressed in growing cells and can be further induced by challenging the cell with H₂O₂. HP II is only expressed in stationary cells and is part of the *rpoS* regulon (Mulvey *et al.*, 1988). Therefore in *rpoS* mutants, HP II is not expressed and the rate at which H₂O₂ decomposition occurs in aged colonies is very much slower in these strains. Typically, bubbling from an aged *rpoS*⁺ colony is immediate and vigorous while that from a mutant colony is slow with a 10-15 second lag between addition of H₂O₂ and beginning of bubbling and a feeble rate of bubbling. It was noticed that the WU3610 strain from laboratory stocks displayed a feeble response upon H₂O₂ addition compared to other strains, such as WP2, which showed the expected vigorous reaction. An independent

isolate of WU3610 was therefore obtained from R. Bockrath (USA) and tested for its hydrogen peroxidase activity. It was found that it displayed the typical *rpoS*⁺ phenotype, viz. aged colonies bubbled vigorously. It was concluded that WU3610 from our stocks had acquired a mutation, either in *rpoS* itself or in the gene coding for HP II. Mutations in *rpoS* would appear to be relatively common (Ivanova *et al.*, 1992; Jishage and Ishihama, 1997; Visick and Clark, 1997). Indeed, *rpoS* mutants can arise and take over stationary cultures suggesting that in some cases the *rpoS* null or defective phenotype is advantageous to the cell (Zambrano *et al.*, 1993). The WU3610 strain with the presumptive defect was named WU3610_(old) while the newly acquired Bockrath isolate was named WU3610_(new).

A comparison was made of the ability of the old and new cultures to undergo starvation-associated mutation, when starved for tyrosine and the results are shown in Figure 5.2.1. In general the WU3610_(old) strain tends to show earlier appearance of the tyrosine independent colonies although there appears to be little difference in the final counts at 21 days (not shown), the results presented show that in these experiments WU3610_(new) exhibited a slower increase in the number of mutants to day 14. Viability of the two strains was also examined Figure 5.2.2. Surprisingly over the first three days, at high cell density, there was no major difference between the two strains although decline in viability of WU3610_(old) would appear to be slightly faster than WU3610_(new). Unfortunately, because of the appearance of slow growing mutants on agar plates, there is no way of accurately determining viability beyond about four days.

Figure 5.2.1



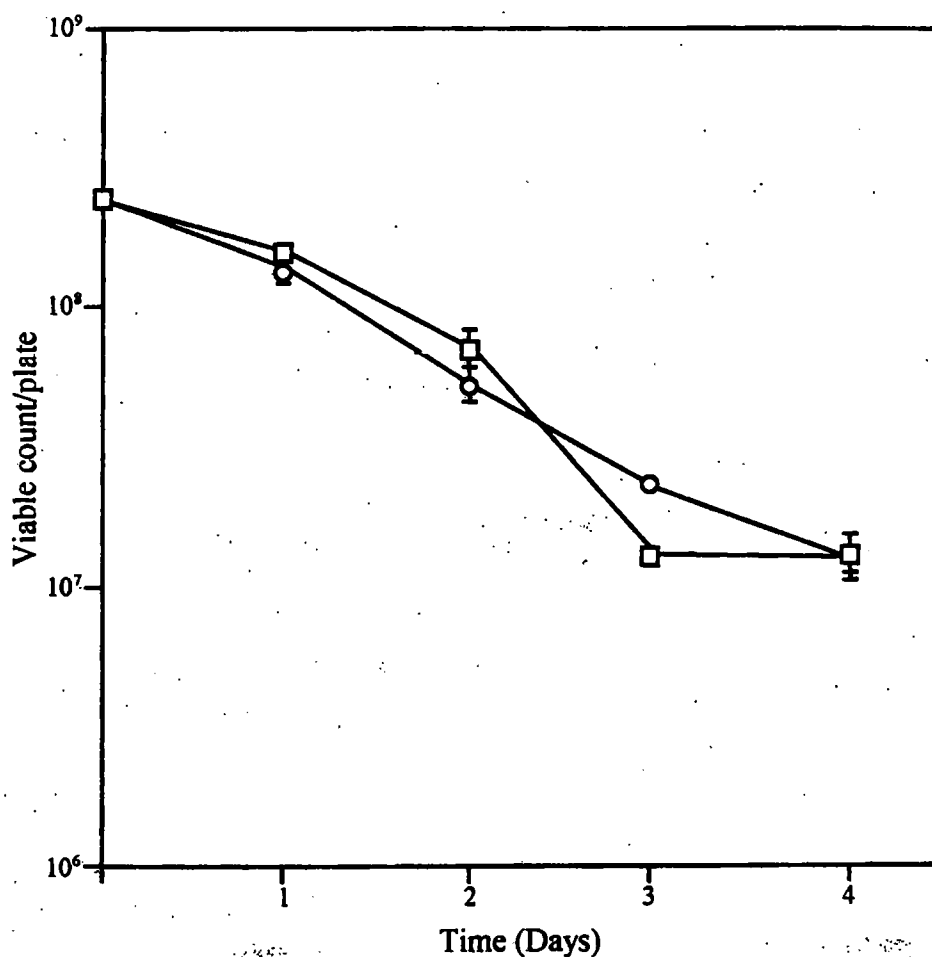
Starvation-associated mutation in WU3610_(old) (●) and WU3610_(new) (○) followed at 27°C. Plates were inoculated with about 3×10^8 bacteria. Points are the mean of at least three experiments.

Therefore, the two strains may have totally different viable counts, when they have been starved for a long period of time, and a lower viability of the presumptive *rpoS*.

Figure 5.2.2

Viability of the *tyrA14* strains WU3610_(old) and WU3610_(new)

on minimal salts agar plus leucine



Viabilities of WU3610_(old) (○) and WU3610_(new) (□) followed over four days at 27°C. Plates were inoculated with about 3×10^8 bacteria. The experiments were performed using whole the plate wash-off technique.

strain WU3610_(old) might account for the decreased rate of occurrence of Tyr⁺ colonies after about day 10 of the starvation experiments.

These results are entirely consistent with those obtained in a *rpoS*::Tn10 derivative of WU3610_(new) (CM1376), reported elsewhere. In this strain, Tyr⁺ colonies also began appearing several days before those in the parent although again the total numbers were very similar after 14 days. The precise nature of the defect in WU3610_(old) has not been determined at a molecular level. However, it would be consistent with the above observations if the mutation was in the *rpoS* gene.

5.3 Appendix III: Gene designations and descriptions

Gene	Product	Description or function
<i>alkA</i>	AlkA	inducible 3-MeA DNA glycosylase II
<i>argE</i>	ArgE	acetylornithine deacetylase, arginine biosynthesis
<i>aroH</i>	AroH	monofunctional chorismate mutase (<i>B.subtilis</i>)
<i>clpA</i>	ClpA	ATP-binding subunit of ClpP protease
<i>clpB</i>	ClpB	ATP-dependent chaperone subunit of ClpP protease
<i>clpP</i>	ClpP	proteolytic subunit of ClpP protease
<i>clpX</i>	ClpX	protease, activator of ClpP
<i>crp</i>	CRP	cAMP receptor protein
<i>dam</i>	Dam	DNA adenine methylase
<i>dcm</i>	Dcm	DNA cytosine methylase
<i>dnaE</i>	Pol III	DNA polymerase III α subunit
<i>dnaQ</i>	ϵ -subunit	DNA polymerase III ϵ -subunit (<i>mutD</i>)
<i>dpsA</i>	Dps	stress induced DNA binding protein
<i>fis</i>	FIS	factor for inversion stimulation, DNA binding protein
<i>gyrA</i>	GyrA	DNA gyrase subunit A
<i>gyrB</i>	GyrB	DNA gyrase subunit B
<i>hisG</i>	HisG	ATP phosphoribosyl transferase, histidine biosynthesis
<i>hnsA</i>	H-NS	histone-like DNA binding protein
<i>ihf</i>	IHF	integration host factor, DNA binding protein
<i>katE</i>	HP I	catalase hydroperoxidase

<i>katG</i>	HP II	bifunctional; catalase-hydrogen peroxidase
<i>lacA</i>	LacA	thiogalactoside acetyltransferase
<i>lacI</i>	LacI	repressor protein of the <i>lac</i> operon
<i>lacY</i>	LacY	galactoside permease
<i>lacZ</i>	LacZ	β -D-galactosidase
<i>leu</i>		leucine biosynthesis
<i>leuB</i>	LeuB	β -isopropylmalate dehydrogenase
<i>lexA</i>	LexA	global regulator of the SOS operon
<i>lig</i>	Lig	DNA ligase
<i>lrp</i>	Lrp	leucine-responsive regulatory protein
<i>mutD</i>	ϵ -subunit	DNA polymerase III ϵ -subunit (<i>dnaQ</i>)
<i>mutH</i>	MutH	methyl-directed mismatch repair
<i>mutL</i>	MutL	methyl-directed mismatch repair
<i>mutM</i>	MutM (Fpg)	formamidopyrimidine-DNA glycosylase
<i>mutS</i>	MutS	methyl-directed mismatch repair
<i>mutT</i>	MutT	8-oxo-dGTP/8-oxo-rGTP hydrolase
<i>mutY</i>	MutY	adenine glycosylase
<i>nfo</i>	Exo IV	endonuclease IV
<i>oriC</i>		chromosomal origin of replication
<i>pheA</i>	P-protein	chorismate mutase/prephenate dehydratase (<i>E.coli</i>)
<i>pheC</i>	PheC	monofunctional prephenate dehydratase (<i>P.aeruginosa</i>)
<i>pnp</i>	PNPase	processive 3'-to-5' phosphorolytic RNA degradation
<i>polA</i>	Pol I	DNA polymerase I

<i>recA</i>	RecA	DNA repair and general recombination
<i>recB</i>	RecB	exonuclease V subunit, DNA repair and recombination
<i>recC</i>	RecC	exonuclease V subunit, DNA repair and recombination
<i>recD</i>	RecD	exonuclease V subunit, DNA repair and recombination
<i>recE</i>	RecE	5' to 3' single-strand exonuclease VIII, in duplex DNA
<i>recF</i>	RecF	DNA repair and recombination
<i>recG</i>	RecG	junction specific DNA helicase
<i>recJ</i>	RecJ	single-strand DNA specific 5' to 3' exonuclease
<i>recQ</i>	RecQ	DNA repair and recombination helicase
<i>rho</i>	Rho (ρ)	Rho transcription termination factor
<i>rna</i>	RNase I	non-specific RNA endoribonuclease
<i>rnb</i>	RNase II	RNA 3'-to-5' exonuclease
<i>rnc</i>	RNase III	double strand endoribonuclease and RNA maturation
<i>rne</i>	RNase E	component of the <i>E.coli</i> RNA degradosome (<i>ams</i>)
<i>rph</i>	RNase PH	RNA phosphorolase
<i>rpoB</i>	RpoB	RNA polymerase β subunit
<i>rpoS</i>	RpoS (σ^S)	stationary phase RNA polymerase sigma factor
<i>rpsL</i>	S12	30S ribosomal subunit protein S12
<i>ruvA</i>	RuvA	Holliday junction recognition and branch migration
<i>ruvB</i>	RuvB	Holliday junction recognition and branch migration
<i>ruvC</i>	RuvC	Holliday structure endonuclease
<i>sbcC</i>	SbcC	processing of DNA hairpins
<i>sbcD</i>	SbcD	processing of DNA hairpins

<i>ssb</i>	SSB	single-strand DNA binding protein
<i>tag</i>	Tag	constitutive 3-MeA DNA glycosylase I
<i>tas</i>	Tas	potential aldo-keto reductase
<i>thr</i>		threonine biosynthesis
<i>topA</i>	Top I	DNA topoisomerase I
<i>trpA</i>	TrpA	tryptophan synthase subunit A, tryptophan biosynthesis
<i>trpE</i>	TrpE	anthranilate synthase component, tryptophan biosynthesis
<i>tyrA</i>	T-protein	chorismate mutase/prephenate dehydrogenase (<i>E.coli</i>)
<i>tyrA</i>	TyrA	monofunctional prephenate dehydrogenase (<i>E.herbicola</i>)
<i>tyrT</i>	tRNA _I ^{Tyr}	tyrosine tRNA _I ^{Tyr} transfer RNA
<i>umuC</i>	UmuC	error-prone DNA repair
<i>umuD</i>	UmuD	error-prone DNA repair
<i>ung</i>	Ung	uracil- <i>N</i> -glycosylase
<i>uvrA</i>	UvrA	UvrABC exinuclease subunit A, DNA excision repair
<i>uvrB</i>	UvrB	UvrABC exinuclease subunit B, DNA excision repair
<i>uvrC</i>	UvrC	UvrABC exinuclease subunit C, DNA excision repair
<i>uvrD</i>	UvrD	DNA helicase II
<i>vsr</i>	VSP	very short patch repair endonuclease
<i>xthA</i>	ExoIII	exonuclease III
<i>ygeD</i>	YgeD	potential, integral membrane protein

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